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(54) Title: ALPHAVIRUS REPLICON VECTOR SYSTEMS

(57) Abstract: The present invention provides compositions useful in and methods for producing populations of infectious, replication-defective alphavirus replicon particles that contain no replication-competent alphavirus particles, as determined by passage on cells in culture. The compositions include helper and replicon nucleic acid molecules that can further reduce the predicted frequency for formation of replication-competent virus and can optimize manufacturing strategies and costs.

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ALPHAVIRUS REPLICON VECTOR SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority from U.S. Provisional Application

10   No. 60/317,722, filed September 6, 2001, which is herein incorporated by reference in

its entirety.

FIELD OF THE INVENTION

The present invention relates to improved constructs for and methods of making

15   recombinant alphavirus particles that are useful in immunotherapies for infectious

diseases and cancer and in the delivery of genes for therapeutic purposes.

BACKGROUND OF THE INVENTION

Alphaviruses are currently being used as a vector platform to develop vaccines

20   for infectious diseases (e.g. see US Patent Nos. 5,792,462; 6,156,558; 5,811,407;

5,789,245; 6,015, 694; 5,739,026; Pushko et al., *Virology* 239(2): 389-401 (1997),

Frolov et al., *J. Virol.* 71(1): 248-258 (1997); Smerdou and Liljestrom, *J. Virol.* 73(2):

1092-1098 (1999). Alphaviruses comprise a genus in the Togaviridae, and members of

the genus are found throughout the world, in both vertebrate and invertebrate hosts.

25   Among the most studied alphaviruses for vector platforms are Venezuelan Equine

Encephalitis (VEE) Virus, Semiliki Forest Virus (SFV), and Sindbis Virus, the

prototype member of the genus. Various constructs have been developed to enhance

immunogenicity and effectiveness in vaccine applications. Many of these constructs

have also been designed to decrease the likelihood of formation of replication-

30   competent virus through recombination. Johnston et al. (US Patent Nos. 5,792,462 and

6,156,558, cited above) recognized the potential for recombination from a single helper

system (in which the complete set of structural proteins of an alphavirus are on one

RNA molecule and the nonstructural proteins and gene of interest are on another

molecule), and thus designed "double-helper" systems that utilized two helper RNAs to

5   encode the structural proteins. Dubensky et al. (US Patent No. 5,789,245) and Polo et

al. (US Patent No. 6,242,259) describe the use of two DNA alphavirus structural

protein expression cassettes to package alphavirus replicons or other alphavirus vectors.

Liljestrom and colleagues have presented data confirming that a "single helper system"

will generate wild-type virus particles through recombination (Bergland, et al. 1993

10   Biotechnology 11(8): 916-920).

By distributing the viral genes among three nucleic acids, two of which

comprise the helper system, as in the above-described art, the theoretical frequency of

recombination that would create a replication-competent virus is reduced significantly

relative to single helper systems. These existing systems include the use of the

15   alphavirus RNA polymerase recognition signals, so that the helper systems can take

advantage of the presence of the alphavirus replication machinery for amplification and

efficient expression of helper functions. However, the presence of the terminal

recognition signals on the helper RNAs also means that recombinants in which the

helper constructs are incorporated into the termini of the replicon RNA by RNA

20   recombination remain replicable.   It is also recognized (e.g. Liljestrom et al. US Patent

No. 6,190,666, Column 17, lines 45-48) that the capsid binding region of nsP1 is

required for the packaging of alphaviral RNA into virus or viral-like particles, and so

removal of this region would result in the reduction of packaging (see also Levis et al.

1986 *Cell* 44:137 and Weiss et al. 1989 *J. Virol.* 63:530).

25   Thus, in existing replicon systems, known packaging signals are typically

included in replicon RNAs and excluded from helper constructs. However, helper

RNAs are nonetheless packaged or copackaged at a lower frequency (Lu and Silver (*J.

Virol Methods* 2001, 91(1): 59-65), and helper constructs with terminal recognition

signals will be amplified and expressed in the presence of a replicon, and potentially

30   yield additional recombination events.

The current preferred dosages for administration of vector replicon particles, as

described by Johnston et al., or recombinant alphavirus particles, as described by

Dubensky et al., are approximately 10^6 to 10^8 particles.   In the case of chimpanzee

administrations, Dubensky et al. have estimated the need for 4 injections, each

5   containing $10^7 - 10^8$ particles, with a Sindbis-HBV vaccine. Such dosages require

large scale manufacturing procedures, and the amounts produced at such scale may be

greater than the predicted frequency for the generation of replication-competent viruses

in these existing systems.

       Thus, there remains a need to further improve systems for manufacturing

10   alphavirus replicon particles to further reduce the predicted frequency for formation of

replication-competent virus, and to optimize manufacturing strategies and costs.

SUMMARY OF THE INVENTION

       The present invention provides improved alphavirus replicon vector systems for

15   producing infectious, replication defective, alphavirus replicon particles in alphavirus-

permissive cells. Encompassed in the invention are improved replicon RNAs and

improved helper nucleic acids for expressing the alphavirus structural proteins. During

the production of recombinant alphavirus particles, the generation of replication-

competent virus particles can occur through recombination alone or through a

20   combination of helper packaging and recombination. Thus, constructs are provided

that eliminate or minimize the occurrence of one or both of these events. In addition,

these constructs are also designed to minimize the manufacturing complexity and cost

of particles made with such constructs. The invention also provides methods of making

recombinant alphavirus particles using the claimed constructs, and pharmaceutical

25   compositions comprising these recombinant alphavirus particles.

       In a first aspect, resolving DNA helpers are provided, namely recombinant

DNA molecules for expressing the alphavirus structural proteins which comprise a

promoter directing transcription of RNA from a DNA sequence comprising, in order (i)

a first nucleic acid sequence encoding at least one alphavirus structural protein, (ii) a

30   second nucleic acid sequence encoding a ribozyme, (iii) a third nucleic acid sequence

encoding an IRES, and (iv) a fourth nucleic acid sequence encoding at least one

alphavirus structural protein, wherein at least one alphavirus structural protein encoded

by the fourth nucleic acid sequence is not encoded by the first nucleic acid sequence.

5   In another embodiment of the resolving DNA helpers of this invention, recombinant DNA molecules for expressing alphavirus structural proteins are provided, comprising a promoter directing the transcription of RNA from a DNA sequence comprising, in order: (i) a first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence, (ii) a second nucleic acid sequence encoding either

10   (a) an RNA sequence that promotes transcription of a protein coding RNA sequence or (b) an IRES; (iii) a third nucleic acid sequence encoding at least one alphavirus structural protein, (iv) a fourth nucleic acid sequence encoding a 3' alphavirus replication recognition sequence, (v) a fifth nucleic acid sequence encoding a ribozyme, (vi)   a sixth nucleic acid sequence encoding an IRES, and (vii) a seventh nucleic acid

15   sequence encoding at least one alphavirus structural protein, wherein at least one alphavirus structural protein encoded by the seventh nucleic acid sequence is not encoded by the third nucleic acid sequence.

        In yet another embodiment of the resolving DNA helpers, the present invention provides a recombinant DNA molecule for expressing alphavirus structural proteins

20   comprising a promoter directing the transcription of RNA from a DNA sequence comprising, in order: (i) a first nucleic acid sequence encoding an IRES, (ii) a second nucleic acid sequence encoding at least one alphavirus structural protein, (iii)a third nucleic acid sequence encoding a ribozyme, (iv) a fourth nucleic acid sequence encoding an IRES, and (v) a fifth nucleic acid sequence encoding at least one

25   alphavirus structural protein, wherein at least one alphavirus structural protein encoded by the fifth nucleic acid sequence is not encoded by the second nucleic acid sequence.

        In a second aspect of the invention, methods for producing infectious, replication-defective alphavirus replicon particles comprising introducing into a population of alphavirus-permissive cells one or more resolving DNA helper(s) of the

30   claimed invention and an alphavirus replicon RNA encoding at least one heterologous RNA such that infectious, replication-defective particles are produced.

        In a third aspect, the present invention provides resolving RNA helpers, namely recombinant DNA molecules for expressing alphavirus structural proteins comprising: (i) a DNA dependent RNA polymerase promoter, (ii) an IRES, (iii) a nucleic acid

5   sequence encoding an alphavirus capsid protein, which is modified to remove the active site of the autoprotease, (iv) a non-autocatalytic protease recognition site, and (v) a nucleic acid sequence encoding at least one alphavirus glycoprotein.

In another embodiment of the resolving RNA helpers of this invention, a recombinant DNA molecule for expressing a resolving RNA helper in vivo is provided

10   comprising (i) a DNA dependent RNA polymerase promoter, (ii) a nucleic acid sequence encoding at least one alphavirus structural protein, (iii) a non-autocatalytic protease recognition site, and (iv) a nucleic acid sequence encoding at least one alphavirus structural protein, wherein the nucleic acid sequences in (ii) and (iv) are not identical. In preferred embodiments of this aspect, the promoter is an RNA polymerase

15   II promoter that is operable in a helper cell.

In yet another embodiment of the resolving RNA helpers of this invention, a recombinant DNA molecule for expressing an RNA helper in vitro is provided, comprising a promoter directing the transcription of RNA from a DNA sequence comprising (i) a first nucleic acid sequence encoding an alphavirus 5' replication

20   recognition sequence, (ii) a transcriptional promoter, (iii) a nucleic acid sequence encoding at least one alphavirus structural protein, (iv) a non-autocatalytic protease recognition site, and (v) a nucleic acid sequence encoding at least one alphavirus structural protein, and (vi) an alphavirus 3' replication recognition sequence, wherein the nucleic acid sequences of (iii) and (v) are not identical.

25   In a fourth aspect, methods for producing infectious, replication-defective alphavirus replicon particles comprising introducing into a population of cells (i) one or more resolving RNA helpers of the present invention, (ii) a protease that recognizes the non-autocatalytic protease recognition site, and (iii) an alphavirus replicon RNA encoding at least one heterologous RNA such that infectious, replication-defective

30   alphavirus replicon particles are produced in the cells. In certain embodiments of this aspect, an RNA polymerase that recognizes the DNA dependent RNA polymerase promoter is also made available in the helper cell along with the recombinant DNA molecule, such that the DNA molecule is transcribed in vivo to produce sufficient alphavirus structural proteins for packaging alphavirus replicon particles.

5   In a fifth aspect of the present invention, a rearranged alphavirus RNA replicon vector is provided comprising in order: (i) a first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence, (ii)   a second nucleic acid encoding alphavirus nonstructural proteins nsp1, nsp2, and nsp3; (iii) either (a) a transcriptional promoter or (b) an IRES, (iv) a nucleic acid encoding at least one heterologous gene of

10   interest, (v) an IRES, (vi) a third nucleic acid encoding an alphavirus nonstructural protein nsp4, and (vii) a fourth nucleic acid encoding a 3' alphavirus replication recognition sequence. In another embodiment of this aspect, a vector construct comprising a 5' promoter operably linked to a cDNA of the rearranged alphavirus replicon RNA is provided.

15   In a sixth aspect, compositions comprising a population of infectious, defective, alphavirus particles, wherein each particle contains an alphavirus replicon RNA comprising a rearranged alphavirus replicon RNA of this invention, and the population has no detectable replication-competent virus, as measured by passage on cell cultures.

        In a seventh aspect, a non-replicating DNA helper is provided, namely a

20   recombinant DNA molecule for expressing alphavirus structural proteins comprising a promoter for directing the transcription of RNA from a DNA sequence operably linked to a DNA sequence encoding a complete alphavirus structural polyprotein-coding sequence, with the proviso that the DNA sequence does not encode alphaviral 5' or 3' replication recognition sequences or an alphavirus subgenomic promoter.

25   In an eighth aspect, a chimeric alphavirus RNA helper is provided, namely a recombinant RNA molecule comprising, in order, a 5' alphavirus replication recognition sequence, a promoter, a nucleic acid encoding at least one alphavirus structural protein, and a 3' alphaviral replication recognition sequence, wherein the promoter is operably linked to the nucleic acid sequence encoding at least one

30   alphavirus structural protein, wherein the transcription-initiating sequence and the RNA polymerase recognition sequence are recognized by the nonstructural viral proteins of Venezuelan equine encephalitis virus, and wherein the transcription-initiating sequence and the RNA polymerase recognition sequence are derived from a virus other than the alphavirus encoding the structural protein.

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5   In a ninth aspect, an alphavirus structural protein expression system based on a
virus other than an alphavirus is provided, comprising two RNA molecules, wherein (a)
a first RNA encodes sequence for viral replicase proteins, and (b) a second recombinant
RNA encodes sequences for (i) the 5' replication recognition sequence for a replication
complex comprising the viral replicase proteins of (a), (ii) one or more alphavirus
10   structural proteins, and (iii) the 3' replication recognition sequence for the replication
complex comprising the viral replicase proteins of (a), wherein, when the first RNA
and the second RNA are introduced into a helper cell, the first RNA replicates the
second RNA, then the second RNA is translated to produce one or more alphavirus
structural proteins. In preferred embodiments of this aspect, the recombinant RNAs
15   and the viral replicase proteins are derived from a nodavirus.

           In a tenth aspect of the invention, methods for producing infectious, replication-
defective alphavirus replicon particles comprising introducing into a population of
alphavirus-permissive cells one or more helper nucleic acids selected from the group
consisting of non-replicating DNA helpers, chimeric alphavirus helpers, and a non-
20   alphavirus based helper system and an alphavirus replicon RNA encoding at least one
heterologous RNA, such that the helpers express all of the alphavirus structural
proteins, producing said alphavirus replicon particles in the cells, and collecting said
alphavirus replicon particles from the cell.

           In an eleventh aspect of this invention, helper cells for expressing infectious,
25   replication-defective, alphavirus particles utilizing any combination of the helpers
disclosed hereinabove are provided comprising, in an alphavirus-permissive cell, (i)
one or more recombinant nucleic acid molecules selected from group consisting of the
resolving DNA helpers, the resolving RNA helpers, the non-replicating DNA helpers,
the chimeric alphavirus helpers and the non-alphavirus helper system, and (ii) an
30   alphavirus replicon RNA encoding at least one heterologous RNA, wherein the one or
more recombinant nucleic acid helpers together encode all alphavirus structural
proteins which assemble together into the alphavirus replicon particles.

5   **DETAILED DESCRIPTION OF THE INVENTION**

Definitions

As used herein, the term "alphavirus" has its conventional meaning in the art, and includes the various species such as VEE, SFV, Sindbis, Ross River Virus, Western Equine Encephalitis Virus, Eastern Equine Encephalitis Virus, Chikungunya,

10   S.A. AR86, Everglades virus, Mucambo, Barmah Forest Virus, Middelburg Virus, Pixuna Virus, O'nyong-nyong Virus, Getah Virus, Sagiyama Virus, Bebaru Virus, Mayaro Virus, Una Virus, Aura Virus, Whataroa Virus, Banbanki Virus, Kyzylagach Virus, Highlands J Virus, Fort Morgan Virus, Ndumu Virus, and Buggy Creek Virus. The preferred alphaviruses used in the constructs and methods of the claimed invention

15   are VEE, S.AAR86, Sindbis (e.g. TR339, see U.S. Patent No. 6,008,035), and SFV.

The terms "5' alphavirus replication recognition sequence" and "3' alphavirus replication recognition sequence" refer to the sequences found in alphaviruses, or sequences derived therefrom, that are recognized by the nonstructural alphavirus replicase proteins and lead to replication of viral RNA. These are sometimes referred

20   to as the 5' and 3' ends, or alphavirus 5' and 3' sequences. In the constructs of this invention, the use of these 5' and 3' ends will result in replication of the RNA sequence encoded between the two ends. These sequences can be modified by standard molecular biological techniques to further minimize the potential for recombination or to introduce cloning sites, with the proviso that they must still be recognized by the

25   alphavirus replication machinery.

The term "minimal 5' alphavirus replication recognition sequence" refers to the minimal sequence that allows recognition by the nonstructural proteins of the alphavirus but does not result in significant packaging/recombination of RNA molecules containing the sequence. In a preferred embodiment, the minimal 5'

30   alphavirus replication recognition sequence results in a fifty to one-hundred fold decrease in the observed frequency of packaging/recombination of the RNA containing that sequence. Packaging/recombination of helpers can be assessed by several methods, e.g. the method described by Lu and Silver (*J. Virol Methods* 2001, 91(1): 59-65).

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5   The terms "alphavirus RNA replicon", "alphavirus replicon RNA" and "alphavirus RNA vector replicon" are used interchangeably to refer to an RNA molecule expressing nonstructural protein genes such that it can direct its own replication (amplification) and comprises, at a minimum, the 5' and 3' alphavirus replication recognition sequences, coding sequences for alphavirus nonstructural

10   proteins, and a polyadenylation tract. It may additionally contain a promoter or an IRES. It may also be engineered to express alphavirus structural proteins. Johnston et al. and Polo et al. (cited in the background) describe numerous constructs for such alphavirus RNA replicons, and such constructs are incorporated herein by reference. Specific embodiments of the alphavirus RNA replicons utilized in the claimed

15   invention may contain one or more attenuating mutations, an attenuating mutation being a nucleotide deletion, addition, or substitution of one or more nucleotide(s), or a mutation that comprises rearrangement or chimeric construction which results in a loss of virulence in a live virus containing the mutation as compared to the appropriate wild-type alphavirus. Examples of an attenuating nucleotide substitution (resulting in an

20   amino acid change in the replicon) include a mutation at nsP1 amino acid position 538, nsP2 amino acid position 96, or nsP2 amino acid position 372 in the alphavirus S.A.AR86.

The terms "alphavirus structural protein/protein(s)" refers to one or a combination of the structural proteins encoded by alphaviruses. These are produced by

25   the virus as a polyprotein and are represented generally in the literature as C-E3-E2-6k-E1. E3 and 6k serve as membrane translocation/transport signals for the two glycoproteins, E2 and E1. Thus, use of the term E1 herein can refer to E1, E3-E1, 6k-E1, or E3-6k-E1, and use of the term E2 herein can refer to E2, E3-E2, 6k-E2, or E3-6k-E2.

30   The term "helper(s)" refers to a nucleic acid molecule that is capable of expressing one or more alphavirus structural proteins.

The terms "helper cell" and "packaging cell" are used interchangeably herein and refer to the cell in which alphavirus replicon particles are produced. The helper cell comprises a set of helpers that encode one or more alphavirus structural proteins.

5   As disclosed herein, the helpers may be RNA or DNA. The cell can be any cell that is

alphavirus-permissive, i.e. cells that are capable of producing alphavirus particles upon

introduction of a viral RNA transcript. Alphavirus-permissive cells include, but are not

limited to, Vero, baby hamster kidney (BHK), 293, 293T, chicken embryo fibroblast

(CEF), and Chinese hamster ovary (CHO) cells. In certain embodiments of the claimed

10   invention, the helper or packaging cell may additionally include a heterologous RNA-

dependent RNA polymerase and/or a sequence-specific protease.

The terms "alphavirus replicon particles", "virus replicon particles" or

"recombinant alphavirus particles" , used interchangeably herein, mean a virion-like

structural complex incorporating an alphavirus replicon RNA that expresses one or

15   more heterologous RNA sequences. Typically, the virion-like structural complex

includes one or more alphavirus structural proteins embedded in a lipid envelope

enclosing a nucleocapsid that in turn encloses the RNA. The lipid envelope is

typically derived from the plasma membrane of the cell in which the particles are

produced. Preferably, the alphavirus replicon RNA is surrounded by a nucleocapsid

20   structure comprised of the alphavirus capsid protein, and the alphavirus glycoproteins

are embedded in the cell-derived lipid envelope. The alphavirus replicon particles are

infectious but replication-defective, i.e. the replicon RNA cannot replicate in the host

cell in the absence of the helper nucleic acid(s) encoding the alphavirus structural

proteins.

25   As described in detail hereinbelow, the present invention provides improved

alphavirus-based replicon systems that reduce the potential for replication-competent

virus formation and that are suitable and/or advantageous for commercial-scale

manufacture of vaccines or therapeutics comprising them. The present invention

provides improved alphavirus RNA replicons and improved helpers for expressing

30   alphavirus structural proteins.

In one embodiment of this invention, a series of "helper constructs", i.e.

recombinant DNA molecules that express the alphavirus structural proteins, is

disclosed in which a single helper is constructed that will resolve itself into two

separate molecules *in vivo*. Thus, the advantage of using a single helper in terms of

10

5   ease of manufacturing and efficiency of production is preserved, while the advantages

of a bipartite helper system are captured in the absence of employing a bipartite

expression system. In one set of these embodiments, a DNA helper construct is used,

while in a second set an RNA helper vector is used. In the case of the DNA helper

constructs that do not employ alphaviral recognition signals for replication and

10   transcription, the theoretical frequency of recombination is lower than the bipartite

RNA helper systems that employ such signals.

In the preferred embodiments for the constructs of this invention, a promoter for

directing transcription of RNA from DNA, i.e. a DNA dependent RNA polymerase, is

employed. In the RNA helper embodiments, the promoter is utilized to synthesize

15   RNA in an *in vitro* transcription reaction, and specific promoters suitable for this use

include the SP6, T7, and T3 RNA polymerase promoters. In the DNA helper

embodiments, the promoter functions within a cell to direct transcription of RNA.

Potential promoters for *in vivo* transcription of the construct include eukaryotic

promoters such as RNA polymerase II promoters, RNA polymerase III promoters, or

20   viral promoters such as MMTV and MoSV LTR, SV40 early region, RSV or CMV.

Many other suitable mammalian and viral promoters for the present invention are

available in the art. Alternatively, DNA dependent RNA polymerase promoters from

bacteria or bacteriophage, e.g. SP6, T7, and T3, may be employed for use *in vivo*, with

the matching RNA polymerase being provided to the cell, either via a separate plasmid,

25   RNA vector, or viral vector. In a specific embodiment, the matching RNA polymerase

can be stably transformed into a helper cell line under the control of an inducible

promoter. Constructs that function within a cell can function as autonomous plasmids

transfected into the cell or they can be stably transformed into the genome. In a stably

transformed cell line, the promoter may be an inducible promoter, so that the cell will

30   only produce the RNA polymerase encoded by the stably transformed construct when

the cell is exposed to the appropriate stimulus (inducer). The helper constructs are

introduced into the stably transformed cell concomitantly with, prior to, or after

exposure to the inducer, thereby effecting expression of the alphavirus structural

proteins. Alternatively, constructs designed to function within a cell can be introduced

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5   into the cell via a viral vector, e.g. adenovirus, poxvirus, adeno-associated virus, SV40,

       retrovirus, nodavirus, picornavirus, vesicular stomatitis virus, and baculoviruses with

       mammalian pol II promoters.

              Once an RNA transcript (mRNA) encoding the helper or RNA replicon vectors

       of this invention is present in the helper cell (either via *in vitro* or *in vivo* approaches, as

10   described above), it is translated to produce the encoded polypeptides or proteins. The

       initiation of translation from an mRNA involves a series of tightly regulated events that

       allow the recruitment of ribosomal subunits to the mRNA. Two distinct mechanisms

       have evolved in eukaryotic cells to initiate translation. In one of them, the methyl-7-

       G(5')pppN structure present at the 5' end of the mRNA, known as "cap", is recognized

15   by the initiation factor eIF4F, which is composed of eIF4E, eIF4G and eIF4A.

       Additionally, pre-initiation complex formation requires, among others, the concerted

       action of initiation factor eIF2, responsible for binding to the initiator tRNA-Met$_i$, and

       eIF3, which interacts with the 40S ribosomal subunit (reviewed in Hershey & Merrick.

       Translational Control of Gene Expression, pp. 33–88. Cold Spring Harbor, NY: Cold

20   Spring Harbor Laboratory Press. 2000.)

              In the alternative mechanism, translation initiation occurs internally on the

       transcript and is mediated by a *cis*-acting element, known as an internal ribosome entry

       site (IRES), that recruits the translational machinery to an internal initiation codon in

       the mRNA with the help of *trans*-acting factors (reviewed in Jackson. Translational

25   Control of Gene Expression, pp. 127–184. Cold Spring Harbor Laboratory Press.

       2000). During many viral infections, as well as in other cellular stress conditions,

       changes in the phosphorylation state of eIF2, which lower the levels of the ternary

       complex eIF2–GTP–tRNA-Met$_i$, results in overall inhibition of protein synthesis.

       Conversely, specific shut-off of cap-dependent initiation depends upon modification of

30   eIF4F functionality (Thompson & Sarnow, Current Opinion in Microbiology 3, 366–

       370, 2000).

              IRES elements bypass cap-dependent translation inhibition; thus the translation

       directed by an IRES is termed "cap-independent". Hence, IRES-driven translation

       initiation prevails during many viral infections, for example picornaviral infection

5   (Macejak & Sarnow. Nature 353, 90–94, 1991). Under these circumstances, cap-

dependent initiation is inhibited or severely compromised due to the presence of small

amounts of functional eIF4F. This is caused by cleavage or loss of solubility of eIF4G

(Gradi et al., Proceedings of the National Academy of Sciences, USA 95, 11089–

.11094, 1998); 4E-BP dephosphorylation (Gingras et al., Proceedings of the National

10   Academy of Sciences, USA 93, 5578–5583. 1996) or poly(A)-binding protein (PABP)

cleavage (Joachims et al., Journal of Virology 73, 718–727, 1999).

          IRES sequences have been found in numerous transcripts from viruses that

infect vertebrate and invertebrate cells as well as in transcripts from vertebrate and

invertebrate genes. Examples of IRES elements suitable for use in this invention

15   include: viral IRES elements from Picornaviruses e.g. poliovirus (PV),

encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV), from

Flaviviruses e.g. hepatitis C virus (HCV), from Pestiviruses e.g. classical swine fever

virus (CSFV), from Retroviruses e.g. murine leukemia virus (MLV), from Lentiviruses

e.g. simian immunodeficiency virus (SIV), or cellular mRNA IRES elements such as

20   those from translation initiation factors e.g. eIF4G or DAP5, from Transcription factors

e.g. c-Myc (Yang and Sarnow, Nucleic Acids Research 25: 2800-2807 1997) or NF-

κB-repressing factor (NRF), from growth factors e.g. vascular endothelial growth factor

(VEGF), fibroblast growth factor (FGF-2), platelet-derived growth factor B (PDGF B),

from homeotic genes e.g. *Antennapedia,* from survival proteins e.g. X-Linked inhibitor

25   of apoptosis (XIAP) or Apaf-1, or chaperones e.g. the immunoglobulin heavy-chain

binding protein BiP (reviewed in Martínez-Salas et al., Journal of General Virology.

82: 973-984, 2001.)

          Preferred IRES sequences that can be utilized in these embodiments are derived

from: encephalomyocarditis virus (EMCV, accession # NC001479), cricket paralysis

30   virus (accession # AF218039), Drosophila C virus accession # AF014388, *Plautia stali*

intestine virus (accession # AB006531), *Rhopalosiphum padi* virus (accession #

AF022937), Himetobi P virus (accession # AB017037), acute bee paralysis virus

(accession # AF150629), Black queen cell virus (accession # AF183905), Triatoma

virus (accession # AF178440), *Acyrthosiphon pisum* virus (accession # AF024514),

5   infectious flacherie virus (accession # AB000906), and Sacbrood virus (accession #

     AF092924). In addition to the naturally occurring IRES elements listed above,

     synthetic IRES sequences, designed to mimic the function of naturally occurring IRES

     sequences, can also be used. In the embodiments in which an IRES is used for

     translation of the promoter driven constructs, the IRES may be an insect IRES or

10   another non-mammalian IRES that is expressed in the cell line chosen for packaging of

     the recombinant alphavirus particles, but would not be expressed, or would be only

     weakly expressed, in the target host. In those embodiments comprising two IRES

     elements, the two elements may be the same or different.

15   **Rearranged Alphavirus RNA Replicon Vectors**

            In all systems described to date which employ alphavirus RNA vector replicons

     to express a heterologous gene of interest, the portion of the alphavirus genome that

     encodes the alphavirus nonstructural proteins (nsps) is maintained intact, i.e. it appears

     in the replicon vectors exactly as it appears in the alphavirus. Disclosed herein is a

20   rearranged alphavirus RNA replicon vector in which the sequence encoding nsp4 has

     been separated from the sequence encoding nsps1-3 and placed under the control of a

     separate translational control element, such as an IRES. Although it is under separate

     control and displaced from the other nonstructural coding sequences, the sequence

     encoding nsp4 is transcribed from the incoming, plus strand of the virus, so that all

25   nonstructural proteins are produced when the replicon is introduced into the helper cell.

     The cassette directing expression of the heterologous gene of interest is placed between

     the two nonstructural gene sequences, which together encode all of the alphavirus

     nonstructural proteins.

            In the existing tripartite alphavirus replicon systems (two helper molecules and

30   the vector replicon), recombination between the helper(s) and the replicon is required to

     generate a replication-competent virus. In these systems, it is generally thought that the

     viral polymerase complex moves between the helper molecules and the replicon

     molecule ("strand-switching"), replicating sequences from all three molecules. In these

     systems, the strand-switching may occur anywhere in the replicon 3' to the 26S

5   promoter directing expression of the heterologous RNA, and still replicate all of the
       nonstructural polyprotein coding region "nsps1-4". In the rearranged replicons claimed
       herein, the theoretical frequency of generation of a replication-competent virus is much
       lower, since any recombination into the heterologous gene region results in the loss of
       the downstream nsp4 gene in the recombinant.

10   Thus, described herein is a recombinant nucleic acid comprising, in order: (i) a
       first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence,
       (ii) a second nucleic acid encoding alphavirus nonstructural proteins nsp1, nsp 2, and
       nsp3; (iii) an IRES, (iv) a nucleic acid sequence encoding at least one heterologous
       gene of interest, (v) an IRES, (vi) a third nucleic acid encoding the alphavirus

15   nonstructural protein nsp4, and (vii) a fourth nucleic acid encoding a 3' alphavirus
       replication recognition sequence. In another embodiment, element (iii) is a
       transcriptional promoter, such as the alphavirus subgenomic promoter (also referred to
       as the 26S promoter or the viral junction region promoter). In certain embodiments,
       this vector replicon RNA is transcribed *in vitro* from a DNA plasmid and then

20   introduced into the helper cell by electroporation. In other embodiments, the vector
       replicon RNA of this invention is transcribed *in vivo* from a DNA vector plasmid that is
       transfected into the helper cell (e.g. see U.S. Patent No. 5,814,482), or it is delivered to
       the helper cell via a virus or virus-like particle.

               The heterologous gene of interest, also referred to herein as a heterologous

25   RNA or heterologous sequence, can be chosen from a wide variety of sequences
       derived from viruses, prokaryotes or eukaryotes. Examples of categories of
       heterologous sequences include, but are not limited to, immunogens, cytokines, toxins,
       therapeutic proteins, enzymes, antisense sequences, and immune response modulators.

               In a preferred embodiment, the 3' alphavirus non-coding sequence used in the

30   replicon construct is approximately 300 nucleotides in length, which contains the 3'
       replication recognition sequence. The minimal 3' replication recognition sequence,
       conserved among alphaviruses, is a 19 nucleotide sequence (Hill et al., Journal of
       Virology, 2693-2704, 1997). The 3' non-coding sequence can be modified through

5   standard molecular biological techniques to minimize the size of the 3' end while
       preserving the replication function.

Resolving DNA Helpers

               Several specific embodiments of the resolving DNA helper constructs are
10   disclosed hereinbelow. In one embodiment, this invention discloses a recombinant
       DNA molecule for expressing alphavirus structural proteins comprising a promoter
       directing the transcription of RNA from a DNA sequence comprising, in order: (i) a
       first nucleic acid sequence encoding at least one alphavirus structural protein, (ii) a
       second nucleic acid sequence encoding a ribozyme, (iii) a third nucleic acid sequence
15   encoding an IRES, and (iv) a fourth nucleic acid sequence encoding at least one
       alphavirus structural protein, wherein at least one alphavirus structural protein encoded
       by the fourth nucleic acid sequence is not encoded by the first nucleic acid sequence.
       In a preferred embodiment, the promoter is a pol II promoter, such as the CMV
       promoter.
20   In a further specific embodiment thereof, the first nucleic acid sequence is
       selected from the group of nucleic acid sequences encoding: capsid, E1 glycoprotein,
       E2 glycoprotein, E1 and E2 glycoprotein, capsid and E1 glycoprotein, or capsid and E2
       glycoprotein. In these specific embodiments, the structural gene nucleic acid
       sequence(s) encoded by the fourth nucleic acid sequence is selected from this same
25   group. In a preferred embodiment, the combination of sequences encoded by the first
       and fourth nucleic acid sequences encompass all the structural proteins required to
       assemble a recombinant alphavirus particle. In a further specific embodiment, one or
       more of the alphavirus structural proteins may encode one or more attenuating
       mutations, for example as defined in U.S. Patent Nos. 5,792,462 and 6,156,558.
30   Specific attenuating mutations for the VEE E1 glycoprotein include an attenuating
       mutation at any one of E1 amino acid positions 81, 272 or 253. Alphavirus replicon
       particles made from the VEE-3042 mutant contain an isoleucine substitution at E1-81,
       and virus replicon particles made from the VEE-3040 mutant contain an attenuating
       mutation at E1-253. Specific attenuating mutations for the VEE E2 glycoprotein

5   include an attenuating mutation at any one of E2 amino acid positions 76, 120, or 209.
Alphavirus replicon particles made from the VEE-3014 mutant contain attenuating
mutations at both E1-272 and at E2-209 (see U.S. Patent No. 5,792,492). A specific
attenuating mutation for the VEE E3 glycoprotein includes an attenuating mutation
consisting of a deletion of E3 amino acids 56-59. Virus replicon particles made from

10   the VEE-3526 mutant contain this deletion in E3 (aa56-59) as well as a second
attenuating mutation at E1-253. Specific attenuating mutations for the S.A.AR86 E2
glycoprotein include an attenuating mutation at any one of E2 amino acid positions
304, 314, 372, or 376.

        In another embodiment, this invention discloses a recombinant DNA molecule

15   for expressing alphavirus structural proteins comprising a promoter directing the
transcription of RNA from a DNA sequence comprising, in order: (i) a first nucleic acid
sequence encoding a 5' alphavirus replication recognition sequence, (ii) a second
nucleic acid sequence encoding either (a) an RNA sequence that promotes transcription
of a protein coding RNA sequence or (b) an IRES; (iii) a third nucleic acid sequence

20   encoding at least one alphavirus structural protein, (iv) a fourth nucleic acid sequence
encoding a 3' alphavirus replication recognition sequence, (v) a fifth nucleic acid
sequence encoding a ribozyme, (vi) a sixth nucleic acid sequence encoding an IRES,
and (vii) a seventh nucleic acid sequence encoding at least one alphavirus structural
protein, wherein at least one alphavirus structural protein encoded by the seventh

25   nucleic acid sequence is not encoded by the third nucleic acid sequence. In a preferred
embodiment, the promoter is a pol II promoter, such as the CMV promoter. In another
embodiment, the promoter is the T7 promoter, and the T7 polymerase is provided in the
packaging cell in any one of the methods described hereinabove. In a specific
embodiment thereof, the third nucleic acid sequence is selected from the group of

30   nucleic acid sequences encoding: capsid, E1 glycoprotein, E2 glycoprotein, E1 and E2
glycoprotein, capsid and E1 glycoprotein, or capsid and E2 glycoprotein. In these
specific embodiments, the structural gene nucleic acid sequence(s) encoded by the
seventh nucleic acid sequence is selected from this same group. In a preferred
embodiment thereof, the combination of sequences encoded by the third and seventh

5   nucleic acid sequences encompass all the structural proteins required to assemble a
     recombinant alphavirus particle. In a further specific embodiment, the sequences
     encoded by the third nucleic acid comprise one or more of the alphavirus glycoprotein
     genes, and the sequence encoded by the seventh nucleic acid comprises the alphavirus
     capsid gene. In a further specific embodiment, one or more of the alphavirus structural

10   proteins may encode one or more attenuating mutations, as defined in U.S. Patent Nos.
     5,792,462 and 6,156,558, and specific examples of which are listed hereinabove.

          In another embodiment, this invention discloses a recombinant DNA molecule
     for expressing alphavirus structural proteins comprising a promoter directing the
     transcription of RNA from a DNA sequence comprising, in order: (i) a first nucleic acid

15   sequence encoding an IRES, (ii) a second nucleic acid sequence encoding at least one
     alphavirus structural protein, (iii) a third nucleic acid sequence encoding a ribozyme,
     (iv) a fourth nucleic acid sequence encoding an IRES, and (v) a fifth nucleic acid
     sequence encoding at least one alphavirus structural protein, wherein at least one
     alphavirus structural protein encoded by the fifth nucleic acid sequence is not encoded

20   by the second nucleic acid sequence. In a preferred embodiment, the promoter is a pol
     II promoter, such as the CMV promoter. In another embodiment, the promoter is the
     T7 promoter, and the T7 polymerase is provided in the packaging cell in any one of the
     methods described hereinabove. In a specific embodiment thereof, the second nucleic
     acid sequence is selected from the group of nucleic acid sequences encoding: capsid,

25   E1 glycoprotein, E2 glycoprotein, E1 and E2 glycoprotein, capsid and E1 glycoprotein,
     or capsid and E2 glycoprotein. In these specific embodiments, the structural gene
     nucleic acid sequence(s) encoded by the fifth nucleic acid sequence is selected from
     this same group. In a preferred embodiment thereof, the combination of sequences
     encoded by the second and fifth nucleic acid sequences encompass all the structural

30   proteins required to assemble a recombinant alphavirus particle. In a further specific
     embodiment, one or more of the alphavirus structural proteins may encode one or more
     attenuating mutations, as defined in U.S. Patent Nos. 5,792,462 and 6,156,558, and
     specific examples of which are listed hereinabove.

5   As described above, the resolving ability of the DNA helper constructs derives from the insertion of a ribozyme. Ribozymes are catalytic RNA molecules possessing the ability to specifically catalyze its own (*cis-*) or other (*trans-*) single-stranded RNA excision (cleavage) following transcription of the RNA *in vivo* from the DNA vector. Ribozymes target a specific RNA sequence, and different ribozymes target different

10   RNA sequences. Through the insertion of nucleotide sequences encoding these ribozymes into the DNA vector, it is possible to engineer molecules that will recognize specific nucleotide sequences within an RNA transcript and cleave it (*Cech, T. Amer. Med. Assn.*, 260:3030, 1988). When a single DNA helper construct as described herein is introduced into a packaging cell, the ribozyme cleaves the single RNA

15   transcript synthesized *in vivo* from the introduced DNA construct at the ribozyme target sequence, resulting in the generation of two separate RNA molecules within the cell, each encoding one or more than one alphaviral structural proteins.

     A wide variety of ribozymes may be utilized within the context of the present invention, including for example, Group I intron ribozymes (Cech et al., U.S. Pat. No.

20   4,987,071); Group II Introns (Michel, et al., EMBO J. 2:33-38 1983), hairpin ribozymes (Hampel et al., Nucl. Acids Res. 18:299-304, 1990, U.S. Pat. No. 5,254,678 and European Patent Publication No. 0 360 257), hammerhead ribozymes (Rossi, J. J. et al., Pharmac. Ther. 50:245-254, 1991; Forster and Symons, Cell 48:211-220, 1987; Haseloff and Gerlach, Nature 328:596-600, 1988; Walbot and Bruening, Nature

25   334:196, 1988; Haseloff and Gerlach, Nature 334:585, 1988), hepatitis delta virus ribozymes (Perrotta and Been, Biochem. 31:16, 1992); *Neurospora* Vakrud satellite (VS) ribozymes (Anderson and Collins, Mol. Cell 5: 4690478, 2000, RNase P ribozymes (Takada et al., Cell 35:849, 1983); as well as other types of ribozymes (see e.g., WO 95/29241, and WO 95/31551). Further examples of ribozymes include those

30   described in U.S. Pat. Nos. 5,116,742, 5,225,337 and 5,246,921.

     The Group I intron ribozyme was the first known ribozyme which was described by Cech and colleagues in Tetrahymena in 1982 (Kruger et al. Cell 31: 147-157, 1992). This ribozyme was found to be involved in the processing of ribosomal RNA (rRNA) through a unique self-splicing manner. The self-splicing of rRNA occurs

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5   by a two step mechanism. First, a guanine nucleotide is added to the 5' end of the intron as the intron-exon junction is being cleaved. Then the freed 5' intron with guanine attacks at the 3' intron-exon junction to release the intron and generate spliced exons (Zaug et al. Nature 324:429-433 1986). Ribonuclease P contains a catalytic RNA and a small subunit protein. It was discovered in bacteria and is able to generate a mature 5'

10   end of tRNA by endonucleocatalytic cleavage of precursor transcripts (Guerrier-Takada et al. Cell 35: 849-857 1983). The mechanism of cleavage by a hammerhead ribozyme has been characterized in the art [see, e.g., Reddy et al., U.S. Pat. No. 5,246,921; Taira et al., U.S. Pat. No. 5,500,357; Goldberg et al., U.S. Pat. No. 5,225,347].

        While an understanding of the precise mechanism of the ribozyme is not

15   necessary to practice the claimed invention, it is generally thought that the ribozyme is attached to the substrate RNA molecule by forming two paired regions via Watson-Crick pairing between the RNA substrate sequence and the two binding regions of the ribozyme. The first deprotonation reaction takes place in the 2' sugar at the 3' side of the substrate cleavage site. This deprotonation causes nucleophilic attack of the

20   adjacent phosphodiester bond and subsequently protonation of the 5' oxyanion cleaving group thereby generating, 2',3'-cyclic phosphate and a 5' hydroxyl terminus.

        Like the hammerhead ribozyme, the hairpin ribozyme was also found in plant viroids, and it acts by a similar mode of action to the hammerhead ribozyme (Feldstein et al. Gene 82:53-61 1989). The design and use of hairpin ribozymes for cleaving an

25   RNA substrate has been described in the art (Hampel et al., U.S. Pat. No. 5,527,895).

        Generally, the targeted sequence of a ribozyme can vary from approximately 3 to 20 nucleotides long. The length of this sequence is sufficient to allow a hybridization with target RNA and disassociation of the ribozyme from the cleaved RNA.

        Haseloff et al (U.S. Patent No. 6,071,730) describe *trans*-splicing ribozymes

30   that also provide precise cleavage sites. *Trans*-splicing ribozymes may be used in alternative embodiments to those described above, in which the element of the recombinant nucleic acid that comprises a ribozyme is substituted therefor with a ribozyme target sequence. The ribozyme itself is then provided in *trans* as a separate DNA or RNA molecule.

5   Thompson et al. (US Patent No. 6,183,959) describe at least seven basic
varieties of enzymatic RNA molecules which are derived from naturally occurring self-
cleaving RNAs. In the embodiments of the invention which employ a ribozyme, an
alternative embodiment employs the use of a pol III promoter, since ribozymes often
have extensive secondary structure that may be more efficiently transcribed by such a
10   promoter.

         As described hereinabove, in one set of embodiments of this invention, one or
more of the nucleic acid sequences encoding the alphavirus structural proteins is/are
placed between the 5' and 3' alphavirus replication recognition sequences, resulting in
amplification of this nucleic acid by the alphavirus replicase proteins. In preferred
15   embodiments, a minimal 5' alphavirus replication recognition sequence is utilized. In a
specific embodiment, all of the alphavirus structural proteins are expressed from a
single promoter. The single transcript is then resolved through a precise cleavage at the
ribozyme site into two replicable (i.e. amplifiable) RNAs (replicable due to the
presence of the 5' and 3' alphavirus replication recognition sequences), each encoding
20   only a subset of the alphavirus structural proteins. These RNAs are then translated
from IRES sequences located 5' to the structural protein coding sequences.

         In a specific, preferred embodiment of the invention, the Hepatitis Delta virus
(HDV) ribozyme is utilized (Wu et al. Science 243:652-655, 1989). The RNA of the
hepatitis delta virus has autocatalytic RNA processing activity similar to that of
25   hammerhead and hairpin ribozymes, and the ribozyme cleavage points of both delta
strands and the domains containing them are clearly defined. The HDV ribozyme is
approximately 80-90 nucleotides in length and only functional *in cis*. Advantages in
using this ribozyme are (i) there are no specific sequence requirements at the 5'
cleavage site and (ii) the cleavage product is generated with defined 3' ends. As
30   described in U.S. Patent No. 5,225,337 (incorporated herein by reference), a preferred
embodiment for an HDV ribozyme is a sequence consisting of at least 18 consecutive
nucleotides from the conserved region of HDV, in which the conserved region of the
HDV RNA is found within either the region of HDV having ribozyme activity between
residues 611 and 771 on the genomic strand or the region between residues 845 and

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5   980 on the complementary anti-genomic strand. The selected sequence having
    ribozyme activity cleaves the target RNA molecule to form a 2',3' cyclic phosphate and
    5' hydroxyl.

        In certain embodiments of this invention, additional sequence alterations are
    made downstream from the ribozyme region. The ribozyme cleavage event results in a
10 clean 3' end, such that the nucleotide sequence remains unchanged. With some
    ribozymes, the 5' end of the downstream molecule resulting from the cleavage event
    may contain residual ribozyme sequence, and the potential secondary structure of this
    residual sequence may have detrimental effects on downstream translational activity,
    e.g. driven by an IRES, or upon any other functional role of the 5' sequence of the
15 downstream fragment. Thus, to minimize the potential for such interference, in some
    embodiments of this invention it may be useful to include a region of non-translated
    irrelevant nucleotide sequence downstream from the ribozyme sequence. In an
    alternative embodiment to generate a clean 5' end, a second antigenomic ribozyme, e.g.
    HDV, can be added downstream from the sense ribozyme. This second ribozyme would
20 be functional only on the negative sense strand, and thus it would generate its clean "3'
    end" on the 5' end of the downstream sequence.

Resolving RNA Helpers

        In another embodiment of resolving helpers of this invention, the helper
25 construct is a DNA helper in which the promoter is a DNA-dependent RNA
    polymerase promoter, such as the T7 polymerase promoter, and this promoter directs
    more than one alphavirus structural protein; preferably capsid and at least one
    glycoprotein. The helper further encodes a protease recognition sequence which is
    inserted in-frame between coding sequences for the alphavirus structural proteins.
30 Thus, disclosed is a recombinant DNA molecule for expressing alphavirus structural
    proteins comprising: (i) a DNA dependent RNA polymerase promoter, (ii) an IRES
    (iii) a nucleic acid sequence encoding at least one alphavirus structural protein, (iv) a
    non-autocatalytic protease recognition site, and (v) a nucleic acid sequence encoding

5   at least one alphavirus structural protein, wherein the nucleic acid sequences in (iii) and

(v) are not identical.

In a separate embodiment, a DNA helper for expressing a resolving RNA helper

in vivo is provided comprising (i) a DNA dependent RNA polymerase promoter, (ii) a

nucleic acid sequence encoding at least one alphavirus structural protein, (iii) a non-

10   autocatalytic protease recognition site, and (iv) a nucleic acid sequence encoding at

least one alphavirus structural protein, wherein the nucleic acid sequences in (ii) and

(iv) are not identical. In a preferred embodiment, the promoter is an RNA polymerase

II promoter, such as the CMV promoter.

In another embodiment, the RNA helper is produced in vitro from a

15   recombinant DNA molecule comprising a promoter directing the transcription of RNA

from a DNA sequence comprising (i) a first nucleic acid sequence encoding an

alphavirus 5' replication recognition sequence, (ii) a transcriptional promoter, (iii) a

nucleic acid sequence encoding at least one alphavirus structural protein, (iv) a non-

autocatalytic protease recognition site, and (v) a nucleic acid sequence encoding at

20   least one alphavirus structural protein, and (vi) an alphavirus 3' replication recognition

sequence, wherein the nucleic acid sequences of (iii) and (v) are not identical. In

preferred embodiments, the promoter is the T7 promoter and the transcription promoter

of element (ii) is an alphavirus subgenomic promoter.

In preferred embodiments of these helpers, the construct produces the

25   polyprotein Capsid-protease site-E2-E1. In other embodiments, the polyprotein is E2-

E1-protease site-Capsid or Capsid-E1-protease site-E2. Signal sequences, such as E3

or 6k, are included as appropriate, with E1 and/or E2 sequences. In those constructs

which encode an alphavirus capsid protein, the capsid protein should be modified to

remove the active site for the autoproteolytic activity of capsid (see U.S. Patent No.

30   5,792,462; Col 11, lines 9-19; Strauss et al. 1990 Seminars in Virology 1:347).

The protease recognition sequence is preferably a relatively rare sequence that

does not occur frequently in the coding sequences of the helper cell. Examples of such

proteases are widely known in the art such as factor Xa, enteropeptidase and thrombin,

but some of these proteases have exhibited lowered specificity for their target

5   recognition/cleavage sites and have cleaved proteins at non-canonical sites. For this

reason, the use of a rare protease with a specific cleavage recognition site which would

be unlikely to be present in the host cell or alphaviral protein repertoire would be a

preferred embodiment in this invention. An example of such a rare protease site is that

of the tobacco etch virus NIa protease (TEV protease). TEV protease cleaves the

10   amino acid sequence ENLYFQG between Q and G with high specificity. In addition,

recent advances in the art have demonstrated the activity of TEV protease can be

increased significantly by a number of methods. One method includes increasing the

solubility of the protease by producing it in the form of a fusion protein. The second

ablates the inherent auto-catalytic function of the protease which severely reduces the

15   functional levels of protease within the cell. Using standard mutagenesis techniques

the autocatalytic domain has been altered and leads to maintenance of high levels of the

protease in its active form (Kapust et al., Protein Engineering, 14:993-1000, 2001). In

the rare event that the consensus cleavage recognition site is present within the

alphaviral vector or the host cell, alternate proteases may be used in this invention.

20   These alternates would most likely be derived from non-mammalian origins to lessen

the chance that the protease would recognize mammalian sequences. Such proteases

would preferably include proteases derived from members of the Potyvirus family of

plant viruses such as Turnip mosaic potyvirus (TuMV) which recognizes an amino acid

sequence XVRHQ where X is any aliphatic amino acid. The invention can also utilize

25   other proteases such as Wheat streak mosaic virus, plum pox virus, potato virus Y,

tobacco vein mottling virus, *Ornithogalum* mosaic virus, yam mosaic virus, shallot

potyvirus, bean yellow mosaic virus, papaya ringspot virus, pea seed-borne mosaic

virus, Johnson grass mosaic virus, rye grass mosaic virus, sweet potato mild mottle

virus, or any other members of the family of C4 unassigned peptidases. The only

30   required feature of the protease in this invention is its restricted ability to recognize

only the target sequence in the vector construct and to lack non-specific protease

activity that could cleave host or other alphaviral sequences.

       In the foregoing embodiments comprising a protease recognition site, the

promoter directs the expression of all encoded alphavirus structural proteins. The

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5   DNA helpers are introduced into the helper cell along with a source of T7 polymerase
    (e.g. a stably transformed expression cassette, a separate expression plasmid, or the
    polymerase protein), a single RNA transcript is produced from the promoter which
    contains the IRES to direct cap-independent translation of a polyprotein, which is then
    cleaved by the protease provided in the helper cell. The RNA helper, transcribed from
10 a DNA helper vector in vitro, is introduced to the helper cell, preferably by
    electroporation, where it is amplified and translated into the polyprotein. As above, the
    protease is provided to the helper cell in any one of several formats, and the polyprotein
    is cleaved in the presence of the protease.

    In a specific embodiment, the protease may be present as a stably transformed
15 cassette in the genome of the helper cell. In this embodiment, the gene encoding the
    protease is preferably under the control of an inducible promoter, such a heat shock or
    metallothionen-responsive promoter. In another embodiment, the protease gene and/or
    the T7 polymerase gene can be present in the helper cell as an alphavirus subgenomic
    expression cassette, comprising an alphavirus 5'end, an alphavirus subgenomic
20 promoter directing expression of the protease, and an alphavirus 3' end. This cassette
    is inducible in the helper cell, being expressed only upon introduction of the alphavirus
    RNA replicon to the cell. Alternatively, the protease may be added to the helper cell
    concomitantly with the RNA helper and the RNA replicon, either as a separately
    translatable RNA or as protein.

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Non-replicating DNA helper

    In another set of embodiments of this invention, a DNA helper that does not
    incorporate the alphavirus replication recognition sequences that allow amplification of
    the RNA encoded between the sequences is disclosed. Lacking such sequences, which
30 can contribute to the frequency of functional recombinant molecules that may be
    generated in the helper cell, a single DNA helper encoding all the alphavirus structural
    proteins necessary to produce recombinant alphavirus particles can minimize the effect
    that packaging/recombination may have in a helper cell. The decrease in
    packaging/recombination detected, as compared to the bipartite RNA system, is at least

5   one order of magnitude lower; in preferred embodiments, it is two, three, or four orders

of magnitude lower.   Thus, another embodiment of the claimed invention comprises a

recombinant DNA for expressing alphavirus structural proteins comprising a promoter

operably linked to a nucleotide sequence encoding all the alphavirus structural proteins.

In a preferred embodiment, the promoter is an RNA polymerase II promoter, such as

10   the CMV promoter.

Preferred methods for introducing this DNA helper to alphavirus-permissive

cells are also disclosed, including the use of cationic lipids, such as FuGene® and

Lipofectamine®, electroporation, or viral vectors. The combination of cell type and

transfection method can be optimized by testing of such combinations according to the

15   methods disclosed herein. In specific embodiments, 293T and Vero cells can be used.

In a preferred embodiment, the DNA helper is introduced prior to the introduction of

the replicon RNA, e.g. thirty minutes, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 24, or 48 hours

prior to electroporation of the replicon RNA into the cells. Any amount of time that

results in no significant decrease in the transfection efficiency of the replicon RNA and

20   allows sufficient packaging of VRPs by the cells is suitable.

In an alternative embodiment, the DNA helpers of this invention may be co-

electroporated into the helper cells with an alphavirus replicon RNA. The parameters

of electroporation are adjusted from those used for solely RNA or DNA electroporation

to optimize the yield of VRPs from the helper cells.

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Chimeric Alphavirus RNA Helpers

In another set of embodiments, an RNA helper which utilizes replication

recognition signals (5' and 3') from alphaviruses other than the alphavirus from which

the structural proteins are derived is disclosed. In these embodiments, the promoter

30   directs the expression of at least one structural protein and the alphavirus 5' and 3' ends

that direct replication of the helper allow amplification, but the frequency of packaging/

recombination by these helpers is reduced, due to the inefficient or complete lack of

recognition of packaging signals of the one alphavirus by the alphavirus structural

proteins of the other alphavirus.   In a preferred embodiment, the RNA helper of this

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5   invention encodes one or more VEE structural proteins and utilizes the replication
     recognition signals from an alphavirus selected from the group consisting of Sindbis
     (particularly TR339), S.A.AR86, Semliki Forest Virus, or Ross River Virus.

     Non-alphavirus RNA Helpers

10   In another set of embodiments of this invention, helper constructs that express
     one or more alphavirus structural proteins are disclosed which utilize viral RNA
     replication machinery derived from a virus other than an alphavirus. Disclosed is an
     alphavirus structural protein expression system, comprising two RNA molecules,
     wherein (a) a first RNA encodes sequences for viral replicase proteins, and (b) a second

15   recombinant RNA encodes sequences for (i) the 5' replication recognition sequence for
     a replication complex comprising the viral replicase proteins of (a), (ii) one or more
     alphavirus structural proteins, and (iii) the 3' replication recognition sequence for the
     replication complex comrising the viral replicase proteins of (a). Following
     introduction of the two RNA molecules into the helper cell, the first RNA replicates the

20   second RNA encoding one or more alphavirus structural proteins, and then the helper
     cell's translational machinery translates the structural protein sequence(s) encoded on
     the second recombinant RNA.

            In a preferred embodiment, the first and second RNA molecules are derived
     from a nodavirus. The *Nodaviridae* are a family of small, non-enveloped, isometric

25   viruses with bipartite positive-sense RNA genomes (Ball & Johnson, 1998 In *The Insect
     Viruses*, pp. 225-267. Edited by L. K. Miller & L. A. Ball. New York:Plenum). The
     nodavirus genomes are among the smallest of all known animal viruses, containing less
     than 5 kb of genetic material. Genomic nodavirus RNA is infectious to insect, plant
     (Selling, B. H., et al., Proc. Natl. Acad. Sci. USA 87:434-438, 1990) and mammalian

30   (Ball, L. A., et al., J. Virol. 66:2326-2334, 1992) cells. Nodaviruses use unique
     regulatory cis-elements, including nodaviral-specific RNA replication (Zhong, W., et
     al., Proc. Natl. Acad. Sci. USA 89:11146-11150, 1992; Ball, L. A. and Li, Y., J. Virol.
     67:3544-3551, 1993; Li, Y. and Ball, L. A., J. Virol. 67:3854-3860, 1993; Ball, L. A., J.
     Virol. 69:720-727, 1995) and packaging signals (Zhong, W., et al., supra, 1992). Both

5   genome segments are capped at their 5' ends but lack poly(A) tails (Newman & Brown,

1976. *Journal of General Virology* 30, 137-140). The smaller segment, referred to as

RNA2, encodes a precursor to the nodavirus coat, or capsid, protein. The larger

segment, referred to as RNA1, encodes the viral portion of the RNA-dependent RNA

polymerase (RdRp), which replicates both RNA1 and 2 (Ball & Johnson, 1998, ibid.).

10   The RNA1 of nodaviruses are notable for their ability to synthesize high cytoplasmic

levels of capped and functional mRNAs, i.e. RNA1 and RNA2. The sequences of

several nodavirus RNA1 molecules have been reported recently, and the RNA2

sequences have been previously disclosed (see Johnson et al. 2001 J. Gen. Virol.

82:1855-1866 and references cited therein, including GenBank accession numbers).

15   Applicants have determined that nodavirus replication and alphavirus

replication can occur at the same time within the same cell. Thus, in one set of

embodiments of this invention, a nodavirus-based alphavirus structural protein

expression system is disclosed, in which RNA1 of a selected nodavirus is supplied to a

cell in which recombinant alphavirus particles are to be packaged, along with an

20   engineered RNA2 from the same or a different nodavirus. The RNA2 is engineered to

remove part of the coding sequence for the nodavirus capsid gene and substitute

therefor the coding sequence for at least one alphavirus structural protein. In a specific

embodiment of the methods of this invention, these two nodavirus RNAs (i.e. RNA1

and engineered RNA2) are co-electroporated with an alphavirus RNA replicon into a

25   cell in which all three RNAs are expressed. In a preferred embodiment, a nodavirus

RNA1, a recombinant nodavirus RNA2 expressing an alphavirus capsid gene, an

alphavirus RNA replicon, and a second helper RNA expressing alphavirus

glycoproteins are co-electroporated into a cell in which all four RNAs can be

expressed, resulting in the packaging of the replicon into recombinant alphavirus

30   particles. Suitable nodaviruses for use in the claimed invention include Nodamura

virus (NoV), Flock House virus (FHV), Black Beetle virus (BBV), Boolarra virus

(BoV), Gypsy moth virus (GMV), and Manawatu virus (MwV). Preferred nodaviruses

are FHV and NoV. In another embodiment, the RNA1 and recombinant RNA2 are

introduced into the helper cell by a virus or a virus-like particle, such as adenovirus,

5   vaccinia, poxvirus, SV-40, adeno-associated virus, retrovirus, nodavirus, picornavirus,

       vesicular stomatitis virus, and baculoviruses with mammalian pol II promoters.

Methods for Making Alphavirus Replicon Particles

             Methods for making alphavirus replicon particles expressing one or more

10   heterologous sequences which utilize the helpers and/or the alphavirus RNA replicons

       of this invention are also disclosed. Using the methods of this invention, preparations

       of alphavirus replicon particles are produced which contain no detectable replication-

       competent alphavirus particles, as determined by passage on alphavirus-permissive

       cells in culture.

15   To make the particles, a helper or combination of helpers is selected such that it

       provides all alphavirus structural proteins necessary to assemble an infectious particle.

       In preferred embodiments, the helper or combination of helpers encode capsid, E1 and

       E2. In an embodiment comprising more than one helper, one of the helpers may be

       selected from the helpers known in the art, e.g. the standard split RNA helpers

20   referenced herein. A helper or combination of helpers comprising at least one

       embodiment of this invention can be used to package any alphavirus replicon RNA. In

       preferred embodiments, the alphavirus replicon RNA is a rearranged alphavirus RNA

       replicon as claimed herein or the standard alphavirus replicon RNA referenced herein.

       In specific, preferred embodiments, the alphavirus replicon particles are VEE-based

25   particles, i.e. the helpers encode VEE alphavirus structural proteins and the replicon

       RNA is derived from VEE.

             Alphavirus replicon particles are prepared according the methods disclosed

       herein in combination with techniques known to those skilled in the art. The methods

       include first introducing the selected helper(s) and an alphavirus replicon RNA

30   encoding one or more heterologous RNAs into a population of alphavirus-permissive

       cells, and then incubating the cells under conditions that allow for the production of

       alphavirus replicon particles. The step of introducing the helper(s) and alphavirus

       replicon RNA to the population of helper cells can be performed by any suitable means,

       as disclosed herein or known to those generally skilled in the art. As described herein,

5   the population of cells may be a stably transformed cell line that provides a promoter, a

trans-acting ribozyme, a protease, or any combination thereof. Alternatively, said

promoters, *trans*-acting ribozymes or proteases may be added to the population of cells

in the form of separately relicable or non-replicable nucleic acids or proteins, as

applicable.

10   Compositions or preparations of alphavirus replicon particles are collected from

the population of helper cells using conventional techniques know to those skilled in

the art, e.g. U.S. Patent No. 5,492,462; 6,156,558; and these compositions are

characterized by the fact that they will contain no detectable, replication-competent

alphavirus particles, as measured by passage on alphavirus-permissive cells in culture.

15   As will be understood by one skilled in the art, there are several embodiments

and elements for each aspect of the claimed invention, and all combinations of different

elements are hereby anticipated, so the specific combinations exemplified herein are

not to be construed as limitations in the scope of the invention as claimed.   If specific

elements are removed or added to the group of elements available in a combination,

20   then the group of elements is to be construed as having incorporated such a change.

           All references cited herein, including publications, patent applications, and

patents, are hereby incorporated by reference to the same extent if each was

individually and specifically indicated to be incorporated by reference, and was

reproduced in its entirety herein.

25

Table 1. PCR primers for Resolving DNA helper cloning

Primer name	Sequence	Amplification product
GP forward	5'CTAGCTAGCTATGTCACTAGTGACCACCATG3' (SEQ ID NO: 1)	VEE glycoprotein
GP reverse	5' GGGCCCTCAATTATGTTTCTGGTTGGT 3' (SEQ ID NO: 2)	VEE glycoprotein
GP-2 forward	5' GCAGAGCTGGTTTAGTGAACCGTATAGGCGGCGCATGAGA GAAGCCCAGACCA 3' (SEQ ID NO: 3)	VEE glycoprotein
GP-2 reverse	5' GCTAGCGCTCTTCCCTTTTTTTTTTTT 3' (SEQ ID NO: 4)	VEE glycoprotein
Capsid	5' GCTCTAGAATGTTCCCGTTCCAGCCAATG 3'	VEE capsid

forward	(SEQ ID NO: 5)	
Capsid reverse	5'GCGTCGACGTCTTGGCCATAGCGGCCGCGGTTACAGACAC ATGGTGGTCACT 3' (SEQ ID NO: 6)	VEE capsid
hdR Forward	5'CGGGTCGGCATGGCATCTCCACCTCCTCGCGGTCCGACCT GGGCATCCGAAGGAGGACGTCGTCCACTCGGATGGCTAAG GGAGAGCTCGC 3' (SEQ ID NO: 7)	Hepatitis delta ribozyme
hdR Reverse	5'TCGAGCGAGCTCTCCCTTAGCCATCCGAGTGGACGACGTC CTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGAT GCCATGCCGACCCGGGCC 3' (SEQ ID NO: 8)	Hepatitis delta ribozyme
CMV forward	5' TAGTTATTAATAGTAATCAATTACGG 3' (SEQ ID NO: 9)	CMV IE promoter
CMV reverse	5'TGGTCTGGGCTTCTCTCATGCGCCGCCTATACGGTTCACTA AACCAGCTCTGC 3' (SEQ ID NO: 10)	CMV IE promoter
d26S forward	5' GGCGCGCCGTCCTCCGATCGTTGTCAGAAG 3' (SEQ ID NO: 11)	CMV IE + VEE 5' NCR
d26S reverse	5' GGCGCGCCTCCGTCAACCGCGTATACATCCTGGTAA 3' (SEQ ID NO: 12)	CMV IE + VEE 5' NCR
E3 forward	5' GGCGCGCCATGTCACTAGTGACCACCATGTG 3' (SEQ ID NO: 13)	E3-E2-6K genes
6K reverse	5' CTCGTAGGCGCCGGCGCCTGCGG 3' (SEQ ID NO: 14)	E3-E2-6K genes
EMCV forward	5' GGCGCGCCAATTCCGCCCCTCTCCCTCCC 3' (SEQ ID NO: 15)	EMCV IRES
EMCV reverse	5' GGCGCGCCTTATCATCGTGTTTTTCAAAG 3' (SEQ ID NO: 16)	EMCV IRES
EMCV-2 forward	5' GCTAGCAATTCCGCCCCTCTCCCTCCC 3' (SEQ ID NO: 17)	EMCV IRES
EMCV-2 reverse	5' GCTAGCTTATCATCGTGTTTTTCAAAG 3' (SEQ ID NO: 18)	EMCV IRES

5

Table 2. PCR primers for Nodavirus RNA helper cloning

Primer name	Primer sequence	Amplification product
Capsid F (MluI)	5' CGACGCGTATGTTCCCGTTCCAGCCAATG 3' (SEQ ID NO: 19)	VEE capsid MluI
Capsid R (MluI)	5' GCACGCGTTTACAGACACATGGTGGTCACT 3' (SEQ ID NO: 20)	VEE capsid MluI, VEE capsid 1, VEE capsid 2
Capsid F1 (NcoI)	5' CCTGCCATGGTATAAATGTTCCCGTTCCAACCA ATG 3' (SEQ ID NO: 21)	VEE capsid 1
Capsid F2 (NcoI)	5' CCTGCCATGGCCCCGTTCCAACCAATG 3' (SEQ ID NO: 22)	VEE capsid 2

EXAMPLES

The following examples are provided to illustrate the present invention, and should not be construed as limiting thereof. In these examples, nm means nanometer, mL means milliliter, pfu/mL means plaque forming units/milliliter, nt means nucleotide(s), PBS means phosphate-buffered saline, VEE means Venezuelan Equine Encephalitis virus, EMC means Encephalomyocarditis virus, BHK means baby hamster kidney cells, GFP means green fluorescent protein, Gp means glycoprotein, CAT means chloramphenicol acetyl transferase, IFA means immunofluorescence assay, and IRES means internal ribosome entry site. The expression "E2 amino acid (e.g., lys, thr, etc.) number" indicates the designated amino acid at the designated residue of the E2 gene, and is also used to refer to amino acids at specific residues in the E1 protein and in the E3 protein.

In the Examples that follow, the starting materials for constructing the various helper plasmids can be selected from any of the following: full-length cDNA clones of VEE, i.e. pV3000, the virulent Trinidad donkey strain of VEE; or any of these clones with attenuating mutations: pV3014 (E2 lys 209, E1 thr 272), p3042 (E1 ile 81), pV3519 (E2 lys 76, E2 lys 209, E1 thr 272) and pV3526 (deletion of E3 56-59, E1 ser 253), which are in the genetic background of Trinidad donkey strain VEE. As described in U.S. Patent No. 5,792,462, these plasmids are digested with restriction enzymes and religated to remove the nonstructural protein coding region. Alternatively, one may start with existing helper plasmids, such as those described in Pushko et al. 1997, Ibid., or as described herein.

Example 1: VEE Replicon Particles

Replicon particles for use as a vaccine or for gene therapy can be produced using the VEE-based vector system (see for example U.S. Patent No. 5,792,462). In these Examples, one or more attenuating mutations (e.g. Johnston and Smith, *Virology* 162(2): 437-443 (1988); Davis et al., *Virology* 171(1): 189-204 (1989); Davis et al., 1990) may have been inserted into VEE sequence to generate attenuated VEE replicon

5   particles (Davis et al., *Virology* 183(1): 20-31 (1991); Davis et al., *Virology* 212(1):

102-110 (1995); Grieder et al., *Virology* 206(2): 994-1006 (1995).

The examples herein describe the construction of an RNA replicon, i.e. an RNA

that self-amplifies and expresses, and one or more helper nucleic acids encoding the

structural proteins to allow packaging. The replicon RNA carries one or more foreign

10   genes, e.g. a gene encoding an immunogen or a reporter gene. The replicon RNA and

the helper nucleic acids (which express the alphavirus structural proteins, as described

hereinbelow) are then introduced into a single cell, i.e. the helper or packaging cell, in

which the replicon RNA is packaged into virus-like particles (herein referred to as

"virus replicon particles" or "VRPs") that are infectious for only one cycle. During the

15   single, infectious cycle, the characteristics of the alphavirus-based vector result in very

high levels of expression of the replicon RNA in cells to which the VRP is targeted,

e.g. cells of the lymph node.

The resulting vaccine vectors are avirulent and provide complete protection

against lethal virus challenge in animals, including but not limited to rodents, horses,

20   nonhuman primates, and humans.

Example 2: Standard VEE Replicons and RNA Helpers

As described in U.S. patent No. 5,792,462, Pushko et al., 1997 (*Virology*

239:389-401), and WO 02/03917 (Olmsted, et al.), a standard alphavirus replicon

25   based on VEE contains the VEE nonstructural genes and a single copy of the 26S

subgenomic RNA promoter followed by a multiple cloning site. In a vaccine construct,

one or more genes encoding an immunogen are inserted into this cloning site. For

purposes of demonstrating the capability of the novel structural protein expression

cassettes of this invention, VEE replicons are constructed by inserting the GFP or CAT

30   gene into this cloning site. Expression of these reporter genes from particles made with

various combinations of the structural protein expression cassettes described herein

demonstrate the utility and novelty of these cassettes.

The standard VEE split RNA helper systems, as described in U.S. Patent Nos.

5,792,462, Pushko et al., 1997 (*Virology* 239:389-401), and PCT publication WO

33

5   02/03917 (Olmsted, et al.), are described herein as "Cap or Gp RNA", "wild-type Gp
       RNA helper", "glycoprotein helper", "Gp helper RNA", "GP-helper", "capsid helper",
       or "C-helper". These RNA helpers are made from DNA plasmids as described in the
       cited references, and these DNA plasmids can be a convenient source for obtaining the
       structural protein coding fragments, e.g. by PCR amplification. Alternatively, these

10   coding fragments can be obtained from full-length clones of VEE or attenuated variants
       thereof (see U.S. Patent No. 5,185,440; U.S. Patent 5,505,947). These standard VEE
       helpers are used in combination with the helper inventions disclosed herein and/or in
       comparative studies with the new systems, as disclosed herein.

15   **Example 3: Rearranged Alphavirus RNA Replicon Vector**
       The nsP4 region was deleted from a standard VEE GFP-expressing replicon
       vector (see Example 2) by digestion with AvrII and ApaI restriction enzymes, followed
       by treatment of the digested DNA with T4 DNA polymerase to generate blunt ends,
       and re-ligation of the DNA to generate pGFPΔnsP4-1. When RNA transcribed *in vitro*

20   from the pGFPΔnsP4-1 DNA plasmid is electroporated into cells, GFP is not expressed.
       However, GFP protein can readily be detected in cells co-electroporated with
       GFPΔnsP4-1 RNA and an unmodified replicon vector RNA. This demonstrates that
       the pGFPΔnsP4-1 vector can be complemented by nsP4 protein provided in *trans* by
       another replicon RNA. This result indicates that the nsp4 gene can function when

25   expressed separately from the other non-structural proteins.
       The nsP4 gene (including a portion of nsP3 to maintain an nsP2 protease
       cleavage site) was then cloned downstream of an EMCV IRES. The nsP4 region was
       PCR amplified from the standard VEE replicon vector with primers nsP34-forward
       (SEQ ID NO: 33) and nsP4-stop (SEQ ID NO: 34) (also, see Table 3). The amplified

30   nsP4 gene fragment was cloned into a transfer vector containing an EMCV IRES, using
       BamHI and XbaI as the 5' and 3' restriction enzyme sites. A second set of primers
       was then used to amplify the EMCV-nsP4 construct from the transfer vector: EMCV
       forward-AscI (SEQ ID NO: 35) and EMCV reverse-AscI (SEQ ID NO: 36), also, see
       Table 3). The EMCV-nsP4 PCR product was digested with AscI restriction enzyme

5   and ligated into AscI linearized pGFPΔnsP4-1 vector DNA, to generate pGFPΔnsP4-

1.1. The cloned nsP4 gene region (including a portion of nsP3 that contains the nsP2

protease site) was sequenced to ensure that no mutations to the nsP4 gene were

introduced during cloning.

RNA transcribed *in vitro* from pGFPΔnsP4-1.1 DNA was electroporated into

10   Vero, BHK, 293T and CEF cells, and GFP protein expression was detected in all cell

types.

Table 3. PCR primers for Rearranged Replicon Vector.

Primer name	Sequence 5'-3'	Region amplified
nsP34 forward	CGGGATCCATGCGGTTTGATGCGGGTGCATA CATC (SEQ ID NO: 33)	VEE nsP4 gene
nsP4 stop	GCTCTAGATTAGCCGTAGAGAGTTATAGGGG (SEQ ID NO: 34)	VEE nsP4 gene
EMCV forward-AscI	TGGCGCGCCGCTCGGAATTCCCCCTCTCCC (SEQ ID NO: 35)	EMCV-nsP4
EMCV reverse-AscI	AGGCGCGCCTTCTATGTAAGCAGCTTGCC (SEQ ID NO: 36)	EMCV-nsP4

15

EXAMPLE 4: Construction of Helpers with
Minimal 5' Alphavirus Replication Recognition Sequence

A.   Constructs for determining the Minimal 5' Untranslated Region (UTR)

When VEE replicon particles (VRP) are inoculated onto fresh cultures of Vero

20   cells, at high multiplicities of infection (MOI), capsid protein can be detected by anti-

capsid immunofluoresence assay (IFA; see Table 5 below). Detection of capsid protein

in VRP infected cells indicates that the capsid gene is present in the VRP. Similar

findings to these have been reported by others (Lu and Silver, 2001, ibid.). This may

occur in at least three ways: 1) as the result of a recombination event between the

25   capsid helper RNA and a replicon RNA, 2) as the result of copackaging of the capsid

helper RNA into a VEE replicon RNA containing particle, or 3) as a result of

35

5   packaging the capsid helper RNA alone into particles (no VEE replicon RNA present).
     VEE helpers previously described in the art (Johnston et al., *ibid.*, Pushko et al., *ibid.*)
     contain 519 nucleotides of the 5' region of VEE RNA. Specifically, this 5' region
     encodes a 45 nt untranslated region (UTR) as well as 474 nt of the nsP1 open reading
     frame (ORF). These helper RNAs were originally designed to remove the VEE
10   nucleotide region thought to be involved in packaging of viral RNAs into particles.
     This design was based on work carried out using Sindbis virus, in which a region of
     nsP1, located approximately 1000 nt into the genome, was thought to encode the
     packaging signal (Bredenbeek, PJ et al., 1993 J Virol 67: 6439-6446; Levis et al 1986
     Cell 44:137-145; Weiss et al. 1989 J Virol 63:5310-8). To further optimize the helper
15   constructs, increasing deletions were made into the 5' UTR to determine the minimal
     sequences required for the helpers to provide (i) acceptable VRP yields and (ii) the
     lowest theoretical frequency for capsid gene copackaging/recombination in VRP
     preparations.

20   **1.   Construction of 5' truncated VEE Capsid Helpers**

     A capsid helper plasmid containing the sequence for the VEE capsid gene was
     constructed as previously described. This construct contains a sequence of 519
     nucleotides, the "5' UTR", located upstream (i.e. 5') from the ATG initiation codon for
     the capsid coding sequence, and is herein referred to as "hcap10".

25

     (a)   PCR-based Construction

     Nine consecutive deletions of approximately 50 nt each were made in the 519 nt
     UTR present in the VEE capsid helper (see Example 2). The following deletions were
     made from the 3' end of the 5' UTR:

30

         Hcap Construct name: 5' UTR nt included:
                 10   1-520
                 1   1-484
                 2   1-435

36

5	3	1-397
	4	1-336
	5	1-294
	6	1-231
	7	1-185
10	8	1-125
	9	1-46

This initial set of capsid helper ("Hcap") constructs was produced using a PCR method that did not require cloning of individual constructs. The procedure was carried out in two separate steps. First, nine reverse primers (Hcap 1-9 reverse) were designed ~50 nt apart, complementary to the 5' UTR up to position 470 of the VEE replicon, and engineered to contain an ApaI restriction site:

	Primer name	SEQ ID NO:
20	48-132.pr2	39
	48-132.pr4	40
	48-132.pr6	41
	48-132.pr8	42
	48-132.pr10	43
25	48-132.pr12	44
	48-132.pr14	45
	48-132.pr16	46
	48-132.pr18	47

The sequences of each of these primers is presented in Table 4. An Hcap forward primer, 13-101.pr1 (SEQ ID NO: 37) (also, see Table 4) was designed so that when it was used in combination with any one of the reverse primers, it would amplify a fragment containing the T7 promoter and the respective 5'UTR deletion. Second, primers were designed to amplify the capsid gene out of the existing capsid helper

37

5   plasmid such that the fragment would have the following composition: 5' ApaI
     restriction site, 26S promoter, capsid gene, 3' UTR – 3'. These primers are 48-132.pr1
     (SEQ ID NO: 48) and 3-8pr4 (SEQ ID NO: 49) (also, see Table 4). The amplified PCR
     products were digested with ApaI enzyme and ligated together at the common ApaI
     site. These ligated DNAs were used as template to PCR amplify each of the Hcap
10   constructs using primers 13-101.pr1 (SEQ ID NO: 37) and 13-101.pr4 (SEQ ID NO:
     38), which flank the 5' and 3' regions of each helper, respectively. The amplified
     Hcap helper DNAs were used to transcribe RNA *in vitro* for use in VRP packaging
     experiments.

15

Table 4. Primers for Hcap construct cloning.

Primer name	Primer sequence 5'-3'	Region amplified
13-101.pr1	CCGGGAAAACAGCATTCCAGGTATTAGA (SEQ ID NO: 37)	Hcap forward
13-101.pr4	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT TTTTTTTTTGAAATATTAAAAACAAAATCCGAT TCGG (SEQ ID NO: 38)	VEE 3' reverse
48-132.pr2	GACGGGCCCCTTGCCCTTCGTAGCGACAC (SEQ ID NO: 39)	Hcap-1 reverse
48-132.pr4	GACGGGCCCAGTTTCCAGGTCAGGGTCGC (SEQ ID NO: 40)	Hcap-2 reverse
48-132.pr6	GACGGGCCCCCTTCATTTTCTTGTCCAATTCCT (SEQ ID NO: 41)	Hcap-3 reverse
48-132.pr8	GACGGGCCCTGCATACTTATACAATCTGTCCG GA (SEQ ID NO: 42)	Hcap-4 reverse
48-132.pr10	GACGGGCCCCGGACAGATACAATGATACTTGT GCT (SEQ ID NO: 43)	Hcap-5 reverse

48- 132.pr12	GACGGGCCCGCCAGATGCGAAAACGCTCTG (SEQ ID NO: 44)	Hcap-6 reverse
48- 132.pr14	GACGGGCCCGCCAGATGCGAAAACGCTCTG (SEQ ID NO: 45)	Hcap-7 reverse
48- 132.pr16	GACGGGCCCTACCTCAAACTGCGGGAAGC (SEQ ID NO: 46)	Hcap-8 reverse
48- 132.pr18	GACGGGCCCTTTTGGGTAGGTAATTGGTCTGG (SEQ ID NO: 47)	Hcap-9 reverse
48-132.pr1	GACGGGCCCCTATAACTCTCTAC (SEQ ID NO: 48)	Capsid forward
3-8pr4	GCAACGCGGGGAGGCAGACA (SEQ ID NO: 49)	Capsid reverse

(b)   *Plasmid Construction of Selected Truncated Helpers*

To construct plasmid versions of selected Hcap clones, each 5' UTR was PCR
amplified as described above, digested with EcoRI and ApaI restriction enzymes, and
then ligated into a standard VEE replicon vector (see Example 2), which was also
digested with EcoRI and ApaI restriction enzymes. The resulting "empty" helper
vectors (Δ-helpers) each contained one of the nine deleted 5' UTR regions. The capsid
gene was then PCR amplified from a standard capsid helper plasmid with primers 48-
132.pr1 and 3-8pr4, as described above, digested with ApaI and NotI restriction
enzymes and ligated into each of the Δ-helpers also linearized with ApaI and NotI
enzymes.

B.   Analysis of Truncated Helper Constructs

Each of the truncated helper constructs was tested separately for its ability to
package replicon RNA expressing an immunogen (e.g. the Gag protein from HIV) into
VRPs. 30 ug of each of the 3 RNAs (i.e. the truncated capsid helper, standard VEE Gp

5   helper, and an HIV-Gag-expressing replicon RNA) were electroporated into CHO or

VERO cells.

1.   **IFA Analysis of Electroporated CHO Cells**

10   Table 5.   IFA Analysis of Electroporated CHO Cells

Sample	GAG	capsid	Gp
Hcap1	>90%	20%	>90%
Hcap2	>90%	15%	>90%
Hcap3	>90%	15%	>90%
Hcap4	>90%	10%	>90%
Hcap5	>90%	5%	>90%
Hcap6	>90%	5%	>90%
Hcap7	>90%	5%	>90%
Hcap8	>90%	<1%	>90%
Hcap9	>90%	<1%	>90%
Hcap10	>90%	>90%	>90%

25   2.   **Packaging/recombination analysis in CHO Cells**

Packaging/recombination studies were carried out on VRP generated in CHO

cells (Table 6). The titer of the resultant GAG VRP and capsid expressing particles

was determined by infection of fresh cells and performing IFA for GAG or capsid

protein.

30   High titered VRP (> 1 x 10^8/ml) were generated in CHO cells using Hcap-1 through

Hcap-6. Most CHO cell VRP preparations generated with these Hcap RNAs had 20 to

>100 fold reduction in capsid copackaging/recombination titer, as compared with

Hcap10 (the standard, "full length 5'UTR" capsid helper).

5   Table 6.

Sample	GAG VRP Titer/ml	capsid titer/ml	fold reduction capsid titer vs hcap10
Hcap1	2.5×10^8	1.4×10^3	23.6
Hcap2	1.0×10^8	1.4×10^3	23.6
Hcap3	1.8×10^8	7.1×10^2	46.5
Hcap4	1.4×10^8	2.6×10^2	126.9
Hcap5	1.1×10^8	1.4×10^3	23.6
Hcap6	1.7×10^8	6.7×10^3	4.9
Hcap7	8.8×10^7	1.4×10^3	23.6
Hcap8	2.8×10^6	nd	nd
Hcap9	7.4×10^6	3.5×10^2	94.3
Hcap10	3.4×10^8	3.3×10^4	0.0

20

nd: not determined

3.   **Packaging/recombination Analysis in Vero Cells using Selected Helpers**

25   Plasmid clones were prepared for the truncated 5' UTRs of Hcap1 through Hcap4, and the clones were sequenced to confirm their identity. Using the Hcap4 truncated helper to provide the capsid protein, VRP were produced following co-electroporation into Vero cells with an HIV-Gag VEE replicon RNA and a standard Gp RNA helper (see Example 2).

30   IFA analysis of Vero cells infected with these VRP at an MOI of ~50-100 (to ensure 100% infection of cells) was carried out 16 hr post infection using an anti-capsid antibody. The number of cells that were IFA-positive for capsid was determined per field at a 10X magnification, and this number was used to calculate a copackaging-recombination titer for capsid on a per milliliter basis. Table 7 provides the results 35   using this truncated helper, as compared with the standard capsid helper ("Hcap10").

Table 7. VRP and Capsid titers in Vero cells using Hcap4 Helper

Sample	GAG VRP Titer/ml	capsid titer/ml	fold reduction capsid titer vs hcap10
Hcap4	9.6×10^8	1.3×10^4	36.2
Hcap10	1.2×10^9	4.7×10^5	0.0

Example 4: T7 polymerase-driven Helpers

A.   T7 polymerase-driven VEE Glycoprotein Helper

The glycoprotein (Gp) genes from the Gp helper are amplified by PCR and cloned downstream from an EMC IRES. The EMC/Gp fragment is then cloned into pCRBlunt (Invitrogen, Carlsbad, CA) under the control of a T7 RNA polymerase promoter. Transfection of this pCRBlunt-EMC/Gp DNA (Fugene 6, Boehringer Mannheim, Indianapolis, IN) into BHK-21 cells expressing T7 RNA polymerase protein resulted in VEE Gp protein expression in cells transfected with the DNA, as determined by IFA.

B.   Resolving T7-polymerase driven VEE DNA Helper

Construction of VEE DNA Resolving Helper

The VEE capsid protein is amplified from standard C helper plasmid (see Example 2) using the VEECapF/XbaI primer (SEQ ID NO: 23) and the VEECapR/BamHI primer (SEQ ID NO: 24) (also, see Table 8). The resulting PCR product is cloned into pCR4-TOPO (InVitrogen Corp., Carlsbad, CA), resulting in pC4-Vcapsid, and orientation is verified by restriction enzyme analysis with SpeI. The VEE glycoprotein genes are PCR amplified from the standard Gp helper plasmid using the VEEGpTEP/BamHI primer (containing the 21nt sequence encoding the tobacco etch virus protease recognition sequence (TEP); SEQ ID NO: 25) and the VEEGpR/EcoRV primer (SEQ ID NO: 26) (also, see Table 8) followed by restriction

5   enzyme digest with BamHI. The pCR4-VCapsid clone is digested with BamHI and

Pmel and ligated with the Bam HI-digested VEEGp PCR product to generate a

structural protein coding cassette with the protease recognition sequence in between the

capsid and Gp sequences. The EMCV IRES is PCR amplified from pIRES using

EMCV-2 forward (SEQ ID NO: 17) and reverse (SEQ ID NO: 18) primers, digested

10   with NheI and cloned into the XbaI restriction enzyme site of pCR4-VSp, and

orientation is verified by PCR analysis with EMCV-2 forward and VEECapR/BamHI.

Table 8. Primers for T7 promoter driven DNA Resolving Helper

Primer	Primer Sequence
VEECapF/XbaI	5'-GTCTAGAATGTTCCCGTTCCAACCAATG-3' (SEQ ID NO: 23)
VEECapR/Bam HI	5'-CGGGATCCCCATTGCTCGCAGTTCTCCGG-3' (SEQ ID NO: 24)
VEEGpTEP/BamHI	5'-CGGGATCCGAAAACCTGTATTTTCAGGGCATGTCACT AGTGACCACCATGTGTCTGCTCGCC-3' (SEQ ID NO: 25)
VEEGpR/EcoR V	5'-CGATATCTCAATTATGTTTCTGGTTG-3' (SEQ ID NO: 26)

15

Example 5: Pol II promoter-driven Non-replicating Helpers

A.   Construction of Helpers

Alphavirus structural protein genes are PCR amplified using gene specific

primers that possess unique restriction enzyme sites and cloned downstream of a DNA

20   Polymerase II promoter for gene expression in transfected cells. In the case of VEE

constructs, capsid and/or Gp genes were PCR amplified from the C-helper or Gp-

helper and cloned into pCDNA-3.1 (InVitrogen, Carlsbad, CA) under the control of a

CMV promoter. None of the clones retained any VEE 5'UTR, 3'UTR or 26S RNA

sequences. The following constructs were generated:

25

5 Name:	Expression Product:
pCDNA-VCap	VEE capsid protein
pCDNA-VcapCleave	VEE capsid protein and 8 amino acids of the E3 protein
pCDNA-VGp(X/X/N)	VEE Gps
pCDNA-VSp	VEE capsid protein and Gps (all VEE structural
10 proteins)	

To construct pCDNA-VSp, the VEE glycoprotein gene was PCR amplified
from the standard Gp helper plasmid (see Example 2) using the VEEGpF/XbaI primer
(SEQ ID NO: 27) and the VEEGpR/NheI primer (SEQ ID NO: 28) (also, see Table 9).
15   The resulting PCR product was digested with XbaI and NheI restriction enzymes and
cloned into the XbaI site of pCDNA3.1(-) to generate pCDNA-VGp(X/N). Orientation
with respect to the CMV immediate early promoter was verified by restriction enzyme
analysis with XbaI and SpeI. The full-length VEE capsid gene was PCR amplified
from the standard C helper plasmid (see Example 2) using the VEECapF/XbaI primer
20   (SEQ ID NO: 29) and the 3-42pr4 primers (SEQ ID NO: 30) (also, see Table 9). This
PCR product was digested with XbaI and ligated into pCDNA-VGp(X/N) at the XbaI
restriction enzyme site to generate pCDNA-VSp. Orientation was verified by
restriction enzyme analysis with SpeI.

25

Table 9. Primers for constructing pCDNA-VSp

Primer	Sequence
VEEGpF/XbaI	5'-GTCTAGAATGTCCCTAGTGACCACCATG-3' (SEQ ID NO: 27)
VEEGpR/NheI	5'-GCGCTAGCGTCAATTATGTTTCTGGTTG-3' (SEQ ID NO: 28)
VEECapF/XbaI	5'-GTCTAGAATGTTCCCGTTCCAACCAATG-3' (SEQ ID NO: 29)
3-42pr4	5'-CAATCGCCGCGAGTTCTATG-3' (SEQ ID NO: 30)

5   **B.   Transfection with pCDNA-VSp**

293T cells in T-175 flasks were transfected with 20μg pCDNA-VSp DNA using

Fugene® 6 (Roche, Indianapolis, IN) or Lipofectamine® 2000 (LF2K; Gibco-BRL,

Gaithersburg, MD) and cells were incubated for 6 or 24h at 37°C with 5% CO_2. Cells

were trypsinized, washed twice in PBS and resuspended at 1.2×10^7 cells/ml in 800μl

10   PBS. Cells were then mixed with 30μg of an alphavirus replicon RNA encoding GFP

and transferred to a 0.4 cm gap electroporation cuvette. The cells and RNA were

pulsed three times at 450 V and 25μF. After 10 minutes at room temperature, the cells

were seeded into T75 flasks with 25mls DMEM + 10% FBS. Samples of each

electroporation were aliquoted to 96 well plates for immunofluorescence analysis and

15   all cells were incubated at 37°C with 5% CO_2.

At approximately 16h post-electroporation, cells in 96 well plates were fixed

with 2% formaldehyde; 0.2% gluteraldehyde and analyzed for GFP protein expression.

Samples were alternatively fixed with MeOH and analyzed by IFA for VEE capsid and

Gp protein expression. Results are summarized in Table 10, as a percentage of the

20   electroporated cells expressing protein.

Table 10. Percentage of cells expressing protein 16h after electroporation.

Helper	Time *	GFP	VEE Capsid	VEE Gp
Cap/Gp RNA	6h	50%	50%	50%
Cap/Gp RNA	24h	80%	>50%	>50%
VSp (Fugene)	6h	80%	<1%	1%
VSp (Fugene)	24h	>80%	<1%	30%
VSp	6h	80%	10%	50%

(LF2K)				
VSp (LF2K)	24h	>80%	1%	80%

* Time elapsed following transformation with helpers that cells were harvested for electroporation with GFP-replicon RNA

Western analysis demonstrates the presence and proper processing of the capsid protein in cells transformed with pCDNA-VSp.

C.   **GFP-expressing VRP Production using DNA Helpers**

Each of the constructs described in (A.) were tested separately or in combination for their ability to package replicon particles expressing a reporter (e.g. GFP protein).

1.   293T cells

293T cells transformed with DNA constructs were electroporated with 30ug GFP-expressing RNA. 30ug of Capsid or Gp helper RNAs were included in electroporation of cells receiving only Gp or Capsid DNAs respectively. At approximately 24h after replicon RNA electroporation as described in (B.), the culture medium was harvested and cell debris was removed by centrifugation in a swinging bucket rotor for 5 min. at 2000 rpm. The supernatant was transferred to new 50ml conical tubes and stored at 4°C. Ten-fold serial dilutions of clarified culture medium, containing alphavirus GFP-VRPs, were prepared, and 30μl of each dilution were inoculated onto Vero cells in 96-well plates. Cells were incubated overnight at 37°C with 5% CO_2 followed by fixation in 2% formaldehyde/0.2% glutaraldehyde. Titrations were determined by counting the number of cells that were GFP positive in 5 fields at the lowest possible dilution. Results are summarized in Table 11 below:

5   Table 11.   GFP VRP Production using DNA Helpers

Sample	Titer/ml VRP	IFA for GFP*	IFA for Capsid*	IFA for Gp*
Control**	1.5×10^6	>80%	20%	20%
VCap	6.4×10^4	50%	20%	10%
CapCleave	1.0×10^5	50%	20%	10%
VGp(X/X/N)	4.3×10^4	50%	20%	20%
VSp	1.9×10^5	50%	1%	30%
CapCleave + VGp(X/X/N)	4.3×10^4	50%	20%	20%

*   Percent of electroporated cells positive for GFP, Capsid or Gp protein expression.

** (Controls = GFP replicon particles generated using VEE Capsid and Gp helper

20   RNAs)

2.   Optimization of DNA Transfection in 293T Cells followed by electroporation using pCDNA-VSp

25   At approximately 24h after replicon RNA electroporation as described in (B.),
the culture medium was harvested and cell debris was removed by centrifugation in a
swinging bucket rotor for 5 min. at 2000 rpm. The supernatant was transferred to new
50ml conical tubes and stored at 4°C. Ten-fold serial dilutions of clarified culture
medium, containing alphavirus GFP-VRPs, were prepared, and 30µl of each dilution
30   were inoculated onto Vero cells in 96-well plates. Cells were incubated overnight at
37°C with 5% CO_2 followed by fixation in 2% formaldehyde/0.2% glutaraldehyde.
Titrations were determined by counting the number of cells that were GFP positive in 5
fields at the lowest possible dilution. Results are summarized in Table 12.

5   Table 12. VRP titers generated using pCDNA-VSp helper

Helper	Time*	Titer (VRP/ml)
Cap/Gp RNA	6h	1.49×10^7
Cap/Gp RNA	24h	5.69×10^7
VSp (Fugene)	6h	2.13×10^5
VSp (Fugene)	24h	2.13×10^6
VSp (LF2K)	6h	9.26×10^5
VSp (LF2K)	24h	2.85×10^6

* Time after transformation with helpers that cells were harvested for subsequent
electroporation with GFP replicon RNA

10   **D.   HIV-Gag expressing VRP Production using pCDNA-VSp Helper**

293T cells in T175 flasks were transfected with pCDNA-VSp DNA, as above,
collected 6h later, and then electroporated with 30µg HIV-Gag replicon RNA (see
Olmsted, et al., WO 02/03917). Electroporated cells were seeded into T75 flasks and
samples were aliquotted into 96-well plates. At 16h post-electroporation, samples of
15   the helper cells in 96-well plates were fixed in methanol and analyzed by IFA for HIV-
Gag, VEE Capsid or VEE Gp protein expression.

Culture medium containing the VRPs released from electroporated cells was
collected, and titrations to determine recombinant alphavirus particle yield were
measured. Titers for HIV-Gag expressing VRP, as well as Capsid and Gp
20   copackaging/single recombinants, were determined by IFA (Table 13).

Table 13. GAG VRP titers and copackaging with VSp DNA helpers.

Helper	HIV-Gag (VRP/ml)	Capsid (FFU/ml)	Gp (FFU/ml)
Cap/Gp RNA	7.75×10^6	1.03×10^3	1.42×10^3
pCDNA-VSp	1.64×10^6	none detected	none detected

5   The results in Table 13 demonstrate that the HIV-Gag VRP titers from the pCDNA-VSp helper were less then 10-fold lower than titers obtained with the bipartite RNA helper system ("Cap/Gp RNA") while the packaging/recombination frequencies were decreased by at least three orders of magnitude.

10   **EXAMPLE 6: Construction of Resolving DNA Helpers**

A.   Resolving DNA Helper A

         To construct this embodiment of the invention, alphavirus capsid and glycoprotein structural protein genes are cloned into two separate positions in the single helper DNA molecule. At least one structural gene, located in the first position, is

15   cloned directly downstream from a DNA dependant RNA polymerase II (pol II) promoter. One or more structural genes, not encoded in the first position, are located in the second position, being positioned downstream of the first position, such that the transcript resulting from the pol II promoter-directed expression contains an IRES element directly 5' to the structural protein gene(s) in the downstream position.

20   One method of construction employs first PCR amplifying the selected alphavirus capsid or glycoprotein structural proteins using structural-gene specific primers that also code for unique restriction enzyme sites. The sequence encoding the structural protein gene(s) located in the first position is cloned directly into the DNA dependent RNA polymerase II (pol II) promoter-based expression vector of choice.

25   The sequence encoding the structural protein gene(s) located in the second position is initially cloned into a transfer vector that contains an IRES sequence such that the transcript eventually resulting from the promoter-directed expression will contain an IRES element directly 5' to the structural protein gene(s) coding sequence in the second position.

30   To insert the ribozyme sequence, overlapping complementary primers that encode a ribozyme are annealed to produce a ribozyme linker sequence with unique 5' and 3' restriction sites at each end. Then, the IRES-structural protein gene fragment is digested out of the transfer vector, and the pol II expression vector is digested at the unique 3' restriction site of the sequence encoding the gene(s) in the first position and

5   at another unique restriction site further downstream that is compatible with the 3' site
     of the IRES-structural protein gene DNA fragment. The construct is completed by
     ligating the IRES-structural protein gene fragment and the pol II expression vector
     together at the compatible 5' and 3' sites at the ends of the ribozyme linker sequence.

10   **Construction of a VEE Helper A**

     The VEE glycoprotein (GP) gene is amplified with GP forward (SEQ ID NO: 1)
     and GP reverse primers (SEQ ID NO: 2) (also, see Table 1) and the VEE capsid (C)
     gene is amplified with C forward (SEQ ID NO: 5) and C reverse primers (SEQ ID NO:
     6) (also, see Table 1) using GP-helper and C-helper plasmids as templates for PCR,
15   respectively (e.g. Pushko et al, 1997). The VEE GP PCR product is digested with NheI
     and ApaI restriction enzymes and ligated into a suitable vector linearized with the same
     restriction enzymes and containing a polII promoter, such as the commercially
     available pCDNA3.1 (Invitrogen, Carlsbad, CA; note that this vector, as well as other
     commercially available vectors, is engineered with a selectable marker for use of the
20   purchased plasmid in mammalian cell culture, but since the claimed inventions do not
     require such a marker, it is removed prior to insertion of the VEE structural protein
     coding sequence(s)). In employing pCDNA3.1, the VEE GP gene is then located
     downstream of the CMV immediate early (IE) promoter, generating pCDNA3.1/sp1.

     The VEE capsid PCR fragment is digested with XbaI and SalI restriction
25   enzymes and ligated into a suitable vector linearized with the same restriction enzymes
     and containing as IRES, such as the commercially available XbaI/SalI linearized pIRES
     DNA (Clontech, Palo Alto, CA). This manipulation generates pIRES/sp2.
     Overlapping complementary primers hdR-Forward (SEQ ID NO: 7) and hdR-Reverse
     (SEQ ID NO: 8) (also, see Table 1), coding for the hepatitis delta ribozyme (hdR), are
30   annealed together to generate an hdR linker sequence with ApaI and XhoI restriction
     sites at the 5' and 3' ends, respectively. The vector portion of the construct is produced
     by digesting pCDNA3.1/sp1 with ApaI and NotI restriction enzymes. A 1502 base
     pair XhoI/NotI IRES/sp2 fragment digested from pIRES/sp2 DNA and the ApaI/XhoI

5   hdR linker fragment are then ligated with ApaI/NotI linearized pCDNA3.1/sp1 to
     generate VEE Helper A.

B.   **Resolving DNA Helpers B and C**

          To construct other embodiments of the invention, an alphavirus structural

10   protein helper vector (e.g. comprising an alphaviral 5' replication recognition sequence,
     either an alphavirus transcriptional promoter (herein Helper B; e.g. an 26S alphavirus
     subgenomic promoter) or an internal ribosome entry site (IRES) (herein Helper C; e.g.
     the EMCV IRES), a nucleic acid sequence encoding the upstream alphavirus structural
     protein gene(s), and an alphavirus 3' replication recognition sequence) is cloned

15   directly into a polII promoter-based expression vector of choice (see Example 5).
     Concomitantly, the nucleic acid sequence encoding the alphavirus structural protein
     gene(s) in the downstream fragment is cloned into a transfer vector containing an IRES
     sequence, as described herein. To assemble Helper B, the IRES-structural protein
     coding sequence fragment is then digested out of the transfer vector and the released

20   fragment is ligated into the promoter expression vector, so that the polII promoter
     directs the expression of the structural proteins in both the upstream and downstream
     positions.

          Helper C is prepared in two steps from Helper B. The first step is to remove the
     26S promoter from Helper B, while introducing a unique restriction site in its place.

25   The second step is to clone an IRES sequence into the unique engineered restriction
     site.

Construction of a VEE Helper B.

          The CMV IE promoter is PCR amplified from pIRES2-DsRed2 (Clontech)

30   using the CMV forward primer (SEQ ID NO: 9) and the CMV reverse primer (SEQ ID
     NO: 10) (also, see Table 1). The VEE 5' non-coding region, 26S promoter, GP gene
     and VEE 3' noncoding region is amplified from a GP-helper plasmid (see Example 4A)
     using the GP-2 forward primer (SEQ ID NO: 3) and the GP-2 reverse primer (SEQ ID
     NO: 4) (also, see Table 1).   The CMV IE PCR product and the GP-helper PCR

5   product have a 53 nt region of homology at their 3' and 5'ends, respectively. This
       region of homology allows an overlapping PCR reaction to produce a gene construct
       containing the CMV IE promoter that initiates transcription at the VEE 5' end of the
       GP-helper sequence. The CMV IE/GP-helper fragment is then digested with NheI
       restriction enzyme. The pol II promoter-based vector pCDNA3.1 is linearized with

10   BglII restriction enzyme and then treated with T4 DNA polymerase to produce a blunt
       end. The vector is further digested with NheI to release the existing CMV IE and T7
       RNA polymerase promoters from the vector. The NheI digested CMV IE/GP-helper
       PCR product is then ligated into the BglII(T4 treated)/NheI digested pCDNA3.1 vector,
       generating pCDNA3.1/sp1.2.   The 1502 base pair XhoI/NotI IRES/sp2 fragment

15   digested from pIRES/sp2 DNA and the ApaI/XhoI hdR linker fragment are then
       ligated with ApaI/NotI linearized pCDNA3.1/sp1.2 to generate VEE Helper B.

Construction of VEE Helper C.

               Removal of the 26S promoter in Helper B is accomplished by PCR, using two

20   sets of primers. The first set of primers (d26S forward (SEQ ID NO: 11) and d26S
       reverse (SEQ ID NO: 12); also, see Table 1) amplify a fragment containing the CMV
       IE and the VEE 5' non-coding region (NCR). The d26S reverse primer anneals
       upstream from the 26S promoter so that it is not included in the PCR product. The
       CMV IE/VEE 5' NCR PCR product encodes a 5' PvuI site, found in the backbone of

25   Helper B DNA, and an engineered unique AscI restriction site at the 3' end of the VEE
       5' NCR. The second set of primers (E3 forward (SEQ ID NO: 13) and 6K reverse
       (SEQ ID NO: 14); also, see Table 1) amplify a product containing the VEE E3-6K
       region of the GP-helper that also does not contain the 26S promoter. The E3-6K
       product contains an engineered 5' AscI site and a 3' unique SgrAI restriction site found

30   in the 6K gene. After PCR amplification, the CMV IE/VEE 5' NCR PCR product is
       digested with PvuI and AscI restriction enzymes, and the E3-6K product is digested
       with AscI and SgrAI restriction enzymes. Helper B DNA (see above) is digested with
       PvuI and SgrAI restriction enzymes to release the CMV IE-VEE 5' NCR-26S-E3-6K
       region. A new Helper is then reconstituted by ligating the PvuI/AscI CMV IE/VEE 5'

5   NCR digested fragment and the AscI/SgrAI E3-6K digested fragment with the

PvuI/SgrAI digested Helper B vector, generating Helper B.2. Helper B.2 is identical to

Helper B except that the 26S promoter has been removed and a unique AscI restriction

site has replaced it.

The EMCV IRES is amplified from the pIRES vector using the EMCV forward

10   primer (SEQ ID NO: 15) and the EMCV reverse primer (SEQ ID NO: 16) which both

contain engineered 5' AscI restriction sites (also, see Table 1). After amplification, the

EMCV IRES PCR product is digested with AscI restriction enzyme and ligated into

AscI linearized Helper B.2 DNA, generating Helper C.

15   C.   **Resolving DNA Helper D**

Another embodiment of this invention can be constructed by modifying Helper

A to contain an IRES element that directs cap-independent translation of the alphavirus

structural proteins in the upstream as well as the downstream location, a construct

referred to herein as Helper D.

20

Construction of a VEE Helper D.

The EMCV IRES is amplified from the pIRES vector using the EMCV-2

forward primer (SEQ ID NO: 17) and the EMCV-2 reverse primer (SEQ ID NO: 18),

each containing engineered 5' NheI restriction sites (also, seeTable 1). After

25   amplification, the EMCV IRES PCR product is digested with NheI restriction enzyme

and ligated into NheI linearized VEE Helper A, generating VEE Helper D.

Example 7: Chimeric Alphavirus RNA Helpers

A. Construction of VEE-SIN Helper

30   The VEE capsid gene was cloned into the XbaI restriction site of the pSINrep5

replicon vector (InVitrogen, Carlsbad, CA). Sindbis-based helpers that contain the

VEE capsid gene were constructed by deleting portions of the Sindbis nsP region from

the pSINrep5/VEEcapsid construct. The pΔSIN/VEEcapsid DNA was prepared by

digesting pSINrep5/VEEcapsid DNA with SmaI and BamHI restriction enzymes,

5   deleting 6567 base pairs (bp) of the nsP genes. A second helper construct

(pΔSIN/VEEcap-2), lacking an additional 667 bp of the nsP region, was prepared by

PCR amplifying a region of pΔSIN/VEEcapsid, using the primers ΔSIN26S/RsrII

forward (SEQ ID NO: 31) and ΔSIN-ApaI/ reverse (SEQ ID NO: 32) (also, see Table

14) and ligating it into RsrII and ApaI linearized pΔSIN/VEEcapsid DNA.

10

Table 14. PCR primers used to generate ΔSIN/VEEcap-2

primer	Sequence 5'-3'	Region amplified
ΔSIN26S/RsrII forward	TTTCGGACCGTCTCTACGGTGGTCCTAAAT AGTC (SEQ ID NO: 31)	nsP and VEE capsid
ΔSIN-ApaI/ reverse	CTGGTCGGATCATTGGGCCC (SEQ ID NO: 32)	nsP and VEE capsid

B.   HIV-Gag expressing VRP Production using a Chimeric Alphavirus Helper

15   The ΔSIN/VEEcapsid or ΔSIN/VEEcap-2 helpers were used to generate VRP in

Vero cells with a VEE Gp RNA helper (as described herein) and a VEE RNA replicon

expressing the HIV Gag gene. The culture medium containing HIV-Gag VRP,

generated using either the ΔSIN/VEEcapsid or ΔSIN/VEEcap-2 helper, was collected,

and the yield of VRPs was determined using an HIV-Gag IFA. Using the

20   ΔSIN/VEEcapsid helper, a VRP titer of 2.6 x 10^6 /ml was obtained; using the

ΔSIN/VEEcap-2 helper, a VRP titer of 1.9 x 10^6 /ml was obtained.

EXAMPLE 8: Construction of Nodavirus-based Helper RNAs

Generally, the constructs are produced as follows. The alphavirus capsid gene

25   is PCR amplified with gene specific primers that introduce restriction enzyme sites.

The PCR amplified product is cloned directly into a Nodavirus RNA2 (generated from

5   either flock house virus (FHV) or nodamura (NoV)), which are digested with
       compatible restriction enzymes.

Construction of Nodavirus VEE Capsid Helpers

              The VEE capsid gene is PCR amplified with the capsid F (MluI) primer (SEQ
10   ID NO: 19) and the capsid R (MluI) primer (SEQ ID NO: 20) that introduce a MluI
       site, (also, see Table 1), using pCDNA VSp (see Example 5) as the template. The VEE
       capsid PCR product is digested with MluI and ligated into the MluI digested FHV
       RNA2 vector and the BssHII digested NoV RNA2. BssHII digestion produces
       compatible cohesive ends with MluI. The resultant clones are sequenced to determine
15   orientation and to verify sequence accuracy.

              Alternatively, the VEE capsid gene can be directionally cloned into the NcoI
       and MluI sites of FHV RNA2 and the NcoI and BssHII sites of NoV RNA2. This NcoI
       site is 5' to the MluI or BssHII sites in the FHV and NoV RNA2, respectively,
       separated by one nucleotide. The VEE capsid gene is PCR amplified with one of two
20   forward primers containing an NcoI site, capsid F1 (NcoI) (SEQ ID NO: 21) and capsid
       F2 (NcoI) (SEQ ID NO: 22), and a reverse primer that contains a MluI site, capsid R
       (MluI) (SEQ ID NO: 20), using pCDNA VSp (see Example 5) as the template. The
       resultant VEE capsid 1 and VEE capsid 2 PCR products are digested with NcoI and
       MluI and ligated into the NcoI/ MluI digested FHV RNA2 vector and the NcoI/BssHII
25   digested NoV RNA2 vector.

              In addition, the VEE glycoprotein gene and the entire VEE structural protein
       gene cassette can be cloned into the FHV RNA2 and the NoV RNA2 expression
       vectors as described above using appropriate primers.

              The nodavirus capsid helper described herein is co-electroporated into VERO
30   cells at 28 C with nodavirus RNA1, a VEE GP helper (e.g. see Pushko, et. al. 1997,
       ibid.) and a VEE RNA replicon expressing the HIV gag protein (see Olmsted et al.,
       WO 02/03917). When the FHV RNA 1 and 2 are used, recombinant alphavirus particle
       yields were 4.3×10^6 per ml. When the NoV RNA 1 and 2 are used, particle yields
       were approximately 2×10^5 per ml.

5

EXAMPLE 9: Production of Replicon Particles using DNA or RNA helpers

The replicon RNA and helper nucleic acids can be introduced to the helper cell

10   by one or more of several different techniques generally known to the art, e.g.

electroporation, transfection (e.g. using calcium phosphate precipitation or nucleic

acids deposited on microbeads or nanoparticles), lipid-mediated DNA uptake, micro- or

nano-projectiles, stable transformation, by incorporation into a viral vector, such as

adenovirus, SV40, poxvirus (e.g. modified vaccinia Ankara), nodavirus or

15   adenoassociated virus, or by coating onto a virus vector, such as adenovirus. When

using a combination of DNA helper nucleic acids and an RNA replicon, these

molecules may be introduced at different times using different techniques. The

difference in timing may provide a period of time for recovery by the cells, and it may

also allow optimization of the differing kinetics of expression from a DNA vector as

20   compared with an RNA vector. The timing can be 30 minutes, 1, 2, 3, 4, 5, 6, 7, 8, 9,

10, 11, 12, or 24 hours between the introduction of each nucleic acid molecule to the

cells. The introduction of DNA helper(s) by a different means and prior to the

electroporation of replicon RNA (and helper RNA, as appropriate) does not have

significant detrimental effects on the efficiency of the RNA electroporation.

25

Transfection of DNA Helpers
Lipid-Mediated

293T, VERO or DF1 cells are first transfected with the DNA helper by

lipofection using Fugene® 6 (Boehringer Mannheim, Indianapolis, IN). Cells are

30   incubated in the presence of Fugene and DNA in medium containing 10% FBS for 1 to

24 hours. After incubation, they are washed with PBS and collected by trypsinization.

5   *Electroporation*

Transfection of DNA into Vero cells using commercially available cationic lipids such as FuGene and Lipofectamine is typically inefficient (~1% efficiency). While these methods may be sufficient for certain applications, electroporation of DNA helpers into Vero cells is a preferred alternative approach. Various parameters of the

10   electroporation process can be optimized to enhance the efficiency of DNA entry into Vero cells.

For example, it is preferable to use purified DNA constructs. For example, plasmid DNA containing a gene under the control of a CMV promoter is first isolated using a high purity maxiprep system (e.g. Qiagen®; Promega Corporation, Madison,

15   WI). Such initially purifed DNA is preferably further purified by phenol extraction in the presence of ethidium bromide and high salt *(reference: Stemmer, WP, Biotechniques. 1991 June; 10(6):726)*. This further purified DNA is resuspended in nuclease free water prior to electroporation.

Another optimization step involves the culturing conditions for Vero cells can

20   be optimized for electroporation of DNA. Vero cells are initially cultured in EMEM medium and grown to late log phase, then harvested by trypsinization, washed twice with Invitrus® (Cell Culture Technologies GmBh, Zurich, Switzerland), and resuspended in 800μl Invitrus medium.

These Invitrus-bathed cells are combined with an optimized amount of purified

25   DNA (typically, concentrations range from 10μg to 200μg) and then transferred to a 0.4cm² gap cuvette. Electroporations is performed using a Biorad Gene Pulser® (BioRad Laboratories, Inc., Hercules, CA), with four pulses at 50μF, over a range of voltages (500-700V). After electroporation, the Vero cells were seeded into 6 well plates and incubated for 24 hours at 37°C with 5% CO_2.

30   In determining the optimized parameters, expression of protein is first analyzed at 16 and 23 hours post-electroporation. The percentage of transfected cells, varying the above parameters, ranged from 1%-40%; an example of an optimized set of parameters is: 150μg purified plasmid DNA and pulsing four times at 650V, 50μF. In

5   addition, there is typically an additional 5-10% increase in expression between 17 and

23 hours post-electroporation.

Electroporation efficiency can also be enhanced by synchronizing the Vero cells

in a specific phase of the cell cycle. In one example, cells are synchronized in the

G2/M phase with aphidicolin (1mg/ml) prior to electroporation (see Golzio, M,

10   Biochem Biophys Acta. 2002 June 13:1563(1-2):23-8). These synchronized cells are

handled as described above and combined with 50μg of plasmid DNA prior to

electroporation. In this example, aphidicolin-treated cells show a two-fold increase in

the percentage of cells transfected, as compared to untreated (unsynchronized) cells.

15   *Electroporation of Alphavirus Replicon RNA*

After introduction of the DNA helpers of this invention, 1.2×10^7 cells are then

electroporated in the presence of a replicon RNA and appropriate helper RNAs (if

required) under the following conditions: 450V (293T) or 850V (VERO and DF1) and

25uF in 0.4cm gap electroporation cuvettes (pulse 3X's with 4 sec. between pulses).

20   Electroporated cells are then allowed to recover for 10 min. before seeding into 25mls

medium containing 10% FBS.

When using RNA replicon and RNA helper combinations, all RNAs may be co-

electroporated into the helper cell at the same time. Methods for RNA electroporation

are as described above. In an alternative embodiment, when introducing the DNA

25   helpers to the helper cell via electroporation, the replicon RNA may be co-

electroporated with the DNA helpers.

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We claim:

1.   A recombinant DNA molecule for expressing alphavirus structural proteins comprising a promoter directing the transcription of RNA from a DNA sequence comprising, in order:

(i)   a first nucleic acid sequence encoding at least one alphavirus structural protein,

(ii)   a second nucleic acid sequence encoding a ribozyme,

(iii)   a third nucleic acid sequence encoding an IRES,

(iv)   a fourth nucleic acid sequence encoding at least one alphavirus structural protein, wherein at least one alphavirus structural protein encoded by the fourth nucleic acid sequence is not encoded by the first nucleic acid sequence.

2.   The recombinant DNA molecule of claim 1 wherein the first and fourth nucleic acid sequences together encode all alphavirus structural proteins.

3.   The recombinant DNA molecule of claim 1 wherein the promoter is an RNA polymerase II promoter.

4.   The recombinant DNA molecule of claim 1 wherein the first nucleic acid sequence encodes the alphavirus glycoprotein E1 and the fourth nucleic acid sequence encodes alphavirus capsid protein.

5.   The recombinant DNA molecule of claim 1 wherein the first nucleic acid sequence encodes the alphavirus glycoprotein E2 and the fourth nucleic acid sequence encodes alphavirus capsid protein.

6.   The recombinant DNA molecule of claim 2 wherein the first nucleic acid
        sequence encodes alphavirus glycoproteins E1 and E2 and the fourth nucleic
        acid sequence encodes alphavirus capsid protein.

7.   The recombinant DNA molecule of claim 2 wherein the first nucleic acid
        sequence encodes alphavirus capsid protein and the fourth nucleic acid
        sequence encodes alphavirus glycoproteins E1 and E2.

8.   The recombinant DNA molecule of claim 1 wherein the alphavirus is selected
        from the group consisting of VEE, Sindbis, TR339, S.A.AR86, Semliki Forest
        Virus, and Ross River Virus.

9.   The recombinant DNA molecule of claim 1 wherein the IRES is derived from a
        gene selected from the group consisting of vertebrate genes, invertebrate genes,
        genes of a virus that infects vertebrate cells, and genes from a virus that infects
        insect cells.

10.   A recombinant DNA molecule for expressing alphavirus structural proteins
        comprising a promoter directing the transcription of RNA from a DNA
        sequence comprising, in order:

        (i)   a first nucleic acid sequence encoding a 5' alphavirus replication
                recognition sequence,
        (ii)   a second nucleic acid sequence encoding an RNA sequence that
                promotes transcription of a protein coding RNA sequence;
        (iii)   a third nucleic acid sequence encoding at least one alphavirus structural
                protein,
        (iv)   a fourth nucleic acid sequence encoding a 3' alphavirus replication
                recognition sequence
        (v)   a fifth nucleic acid sequence encoding a ribozyme,

(vi)   a sixth nucleic acid sequence encoding an IRES,

(vii)   a seventh nucleic acid sequence encoding at least one alphavirus structural protein,

wherein at least one alphavirus structural protein encoded by the seventh nucleic acid sequence is not encoded by the third nucleic acid sequence.

11.   A recombinant DNA molecule for expressing alphavirus structural proteins comprising a promoter directing the transcription of RNA from a DNA sequence comprising, in order:

(i)   a first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence,

(ii)   a second nucleic acid sequence encoding an IRES,

(iii)   a third nucleic acid sequence encoding at least one alphavirus structural protein,

(iv)   a fourth nucleic acid sequence encoding a 3' alphavirus replication recognition sequence,

(v)   a fifth nucleic acid sequence encoding a ribozyme,

(vi)   a sixth nucleic acid sequence encoding an IRES,

(vii)   a seventh nucleic acid sequence encoding at least one alphavirus structural protein,

wherein at least one alphavirus structural protein encoded by the seventh nucleic acid sequence is not encoded by the third nucleic acid sequence.

12.   The recombinant DNA molecule of claim 10 or 11 wherein the third and the seventh nucleic acid sequences together encode all alphavirus structural proteins.

13. The recombinant DNA molecule of claim 10 or 11 wherein the third nucleic acid sequence encodes alphavirus glycoproteins E1 and E2 and the seventh nucleic acid sequence encodes alphavirus capsid protein.

14. The recombinant DNA molecule of claim 10 or 11 wherein the third nucleic acid sequence encodes alphavirus capsid protein and the seventh nucleic acid sequence encodes alphavirus glycoproteins E1 and E2.

15. The recombinant DNA molecule of claim 10 wherein the second nucleic acid sequence is an alphavirus subgenomic promoter.

16. The recombinant DNA molecule of claim 10 or 11 wherein the first nucleic acid sequence consists of a minimal 5' alphavirus replication recognition sequence.

17. The recombinant DNA molecule of claim 10 or 11 wherein the promoter is an RNA polymerase II promoter.

18. A method of producing infectious, replication-defective alphavirus replicon particles comprising introducing into a population of cells (i) a recombinant DNA molecule of any one of claims 2, 6, 7, 10, or 11 encoding all the alphavirus structural proteins, and (ii) an alphavirus replicon RNA encoding at least one heterologous RNA, such that infectious, replication-defective alphavirus replicon particles are produced.

19. A method of producing infectious, replication-defective alphavirus replicon particles comprising introducing into a population of cells (i) two recombinant DNA molecules from any combination of claims 1, 4, 5, 10, or 11, and (ii) an alphavirus replicon RNA encoding at least one heterologous RNA, wherein the two recombinant molecules together encode all alphavirus structural proteins,

such that infectious, replication-defective alphavirus replicon particles are
produced.

20.   A recombinant DNA molecule for expressing alphavirus structural proteins
      comprising a promoter directing the transcription of RNA from a DNA
      sequence comprising, in order:

      (i)   a first nucleic acid sequence encoding an IRES,

      (ii)   a second nucleic acid sequence encoding at least one alphavirus
              structural protein,

      (iii)   a third nucleic acid sequence encoding a ribozyme,

      (iv)   a fourth nucleic acid sequence encoding an IRES,

      (v)   a fifth nucleic acid sequence encoding at least one alphavirus structural
              protein,

      wherein at least one alphavirus structural protein encoded by the fifth nucleic
      acid sequence is not encoded by the second nucleic acid sequence.

21.   The recombinant DNA molecule of claim 20 wherein the second and fifth
      nucleic acid sequences together encode all alphavirus structural proteins.

22.   The recombinant DNA molecule of claim 20 wherein the promoter is for an
      RNA polymerase selected from the group consisting of T7, T3, SP6 and
      eukaryotic and viral RNA polymerase IIs.

23.   A method of producing infectious replication-defective alphavirus replicon
      particles comprising introducing into a population of cells (i) one or more
      recombinant DNA molecules of claim 20, and (ii) an alphavirus replicon RNA
      encoding at least one heterologous RNA wherein the recombinant DNA

molecules encode all alphavirus structural proteins such that infectious replication-defective alphavirus replicon particles are produced.

24.   The method of claim 23 further comprising introducing into the population of cells a DNA dependent RNA polymerase that recognizes the promoter.

25.   The method of claim 23 wherein the RNA polymerase is introduced to the population of cells on a separate plasmid encoding an RNA polymerase II promoter operably linked to and directing the expression of a nucleic acid sequence encoding the RNA polymerase.

26.   The method of claim 23 wherein the population of cells is derived from a cell line stably transformed with a gene encoding the RNA polymerase and wherein the RNA polymerase is introduced by expression from the stably transformed gene.

27.   The method of any one of claims 24, 25 or 26 wherein the RNA polymerase is T7 and the promoter is the T7 promoter.

28.   A recombinant DNA molecule for expressing alphavirus structural proteins comprising:

(i)   a DNA dependent RNA polymerase promoter,

(ii)   an IRES,

(iii)   a nucleic acid sequence encoding an alphavirus capsid protein, which is modified to remove the active site of the autoprotease,

(iv)   a non-autocatalytic protease recognition site, and

(v)   a nucleic acid sequence encoding at least one alphavirus glycoprotein.

29.   The recombinant DNA molecule of claim 28 wherein the RNA polymerase promoter is the T7 promoter.

30.   The recombinant RNA molecule of claim 28 wherein at least one of the nucleic acid sequences of (ii) and (iv) include an attenuating mutation.

31.   The recombinant RNA molecule of claim 28 wherein the non-autocatalytic protease recognition site is from tobacco etch virus.

32.   A method of producing infectious, replication-defective alphavirus replicon particles comprising introducing into a population of cells (i) one or more recombinant DNA molecules of claim 28, (ii) an RNA polymerase that recognizes the DNA dependent RNA polymerase promoter, (iii) a protease that recognizes the non-autocatalytic protease recognition site, and (iv) an alphavirus replicon RNA encoding at least one heterologous RNA such that infectious, replication-defective alphavirus replicon particles are produced.

33.   A recombinant nucleic acid molecule comprising, in order:

        (i)   a first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence,

        (ii)   a second nucleic acid encoding alphavirus nonstructural proteins nsp1, nsp2, and nsp3;

        (iii)   a transcriptional promoter,

        (iv)   a nucleic acid encoding at least one heterologous gene of interest,

        (v)   an IRES,

        (vi)   a third nucleic acid encoding an alphavirus nonstructural protein nsp4, and

        (vii)   a fourth nucleic acid encoding a 3' alphavirus replication recognition sequence.

34.   A recombinant nucleic acid comprising, in order:

    (i)   a first nucleic acid sequence encoding a 5' alphavirus replication recognition sequence,

    (ii)   a second nucleic acid encoding alphavirus nonstructural proteins nsp1, nsp 2, and nsp3;

    (iii)   an IRES, .

    (iv)   a nucleic acid encoding at least one heterologous gene of interest,

    (v)   an IRES,

    (vi)   a third nucleic acid encoding the alphavirus nonstructural protein nsp4, and

    (vii)   a fourth nucleic acid encoding a 3' alphavirus replication recognition sequence.

35.   The recombinant nucleic acid of claim 33 wherein the nucleic acid is RNA.

36.   The recombinant nucleic acid of claim 34 wherein the nucleic acid is RNA.

37.   The recombinant nucleic acid of claim 33 or 34 wherein the first, second, third and fourth nucleic acids are derived from VEE, Semliki Forest Virus, or Sindbis Virus.

38.   An alphavirus vector construct comprising a 5' promoter operably linked to a cDNA of the RNA of claim 35.

39.   An alphavirus vector construct comprising a 5' promoter operably linked to a cDNA of the RNA of claim 36.

40.   A composition comprising a population of infectious, defective, alphavirus replicon particles, wherein each particle contains an alphavirus replicon RNA comprising the nucleic acid of claim 35, and the population has no detectable replication-competent virus, as measured by passage on cell cultures.

41.   A composition comprising a population of infectious, defective, alphavirus replicon particles, wherein each particle contains an alphavirus replicon RNA comprising the nucleic acid of claim 36, and the population has no detectable replication-competent virus, as measured by passage on cell cultures.

42.   The recombinant nucleic acid of claim 33 wherein the transcriptional promoter is an alphavirus 26S subgenomic promoter.

43.   A recombinant DNA molecule for expressing alphavirus structural proteins comprising a promoter for directing the transcription of RNA from a DNA sequence operably linked to a DNA sequence encoding a complete alphavirus structural polyprotein-coding sequence, with the proviso that the DNA sequence does not encode alphaviral 5' or 3' replication recognition sequences or an alphavirus subgenomic promoter.

44.   A helper cell for expressing an infectious, replication-defective, alphavirus replicon particle comprising, in an alphavirus-permissive cell,
       (i)   one or more recombinant nucleic acid molecules selected from any of claims 1, 10, 11, 20, or 28; and
       (ii)   an alphavirus replicon RNA encoding at least one heterologous RNA, wherein the one or more recombinant nucleic acids together encode all alphavirus structural proteins which assemble together into the alphavirus replicon particles.

45.   A helper cell for expressing an infectious, replication-defective, alphavirus replicon particle, selected from the group consisting of 293, 293T, BHK, Vero, CHO, CEF and DF-1, comprising:

    (i)   one or more recombinant nucleic acid molecules selected from any of claims 1, 10, 11, 20, or 28; and

    (ii)   an alphavirus replicon RNA encoding at least one heterologous RNA, wherein the one or more recombinant nucleic acids together encode all alphavirus structural proteins which assemble together into the alphavirus replicon particles.

46.   A recombinant RNA molecule comprising, in order, a 5' alphavirus replication recognition sequence, a promoter, a nucleic acid encoding at least one alphavirus structural protein, and a 3' alphaviral replication recognition sequence, wherein the promoter is operably linked to the nucleic acid sequence encoding at least one alphavirus structural protein, wherein the transcription-initiating sequence and the RNA polymerase recognition sequence are recognized by the nonstructural viral proteins of Venezuelan Equin Encephalitis virus, and wherein the transcription-initiating sequence and the RNA polymerase recognition sequence are derived from a virus other than the alphavirus encoding the structural protein.

47.   The recombinant RNA molecule of claim 46 wherein the alphavirus structural protein is selected from the group consisting of capsid, capsid-E1, capsid-E2, or E1-E2.

48.   The recombinant RNA molecule of claim 46 wherein the nucleic acid sequence encoding the at least one alphavirus structural protein comprises one or more attenuating mutations.

49.   The recombinant RNA molecule of claim 46 wherein the nucleic acid sequence encoding at least one alphavirus structural protein encodes all the structural proteins of said alphavirus.

50.   The recombinant RNA molecule of claim 46 wherein the 5' and 3' replication recognition sequences are derived from a virus selected from the group consisting of Sindbis Virus, Semliki Forest Virus, and Ross River virus

51.   A cDNA molecule encoding the recombinant RNA molecule of any one of claims 46, 47, 48, 49, or 50.

52.   A method of making infectious, defective alphavirus particles, comprising introducing into a population of cells the following:

        (a)   a recombinant RNA molecule according to claim 47;

        (b)   an alphavirus replicon RNA;

        (c)   a second recombinant RNA molecule according to claim 47;

        wherein the recombinant RNA molecules of (a) and (c) express all of the alphavirus structural proteins, producing said alphavirus particles in the cells, and collecting said alphavirus particles from the cells.

53.   The method according to claim 52, wherein the 5' and 3' sequences of the alphavirus replicon RNA of (b) are from a different alphavirus than the 5' and 3' sequences in the molecule of (a).

54.   An alphavirus structural protein expression system, comprising two RNA molecules, wherein

        (a)   a first RNA encodes sequence for viral replicase proteins, and

(b)   a second recombinant RNA encodes sequences for (i) the 5' replication

recognition sequence for a replication complex comprising the viral

replicase proteins of (a), (ii) one or more alphavirus structural proteins,

and (iii) the 3' replication recognition sequence for the replication

complex comprising the viral replicase proteins of (a),

wherein, when the first RNA and the second RNA are introduced into a helper

cell, the first RNA replicates the second RNA, then the second RNA is

translated to produce one or more alphavirus structural proteins.

55.   The expression system of claim 54 wherein the viral replicase proteins are from
a nodavirus.

56.   The expression system of claim 55 wherein the nodavirus is selected from the
group consisting of flock house virus and nodamura virus.

57.   The expression system of claim 54 wherein the alphavirus structural protein is
capsid.

58.   The expression system of claim 54 wherein the alphavirus is selected from the
group consisting of VEE, SA.A.R.86, TR339, Sindbis, Ross River, and Semliki
Forest.

59.   The expression system of claim 54 wherein the RNA molecules are introduced
to the helper cell by a method selected from the group consisting of virus and
electroporation.

60.   A helper cell containing the expression system of claim 54.

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61.   The expression system of claim 59 wherein the virus is selected from the group
       consisting of adenovirus, vaccinia, poxvirus, SV-40, adeno-associated virus,
       retrovirus, nodavirus, picornavirus, vesicular stomatitis virus, and baculoviruses
       with mammalian pol II promoters.

62.   The expression system of claim 54 wherein the host cell is selected from the
       group of cells consisting of Vero, Baby Hamster Kidney, Chinese Hamster
       Ovary, chicken embryo fibroblasts, DF-1, 293, 293T, and mosquito cells.

63.   A method for making infectious, replication-defective alphavirus replicon
       particles, comprising introducing into a population of cells the following:

       (a)   an alphavirus structural protein expression system according to claim
       54;
       (b)   an alphavirus replicon RNA encoding at least one heterologous RNA;
       (c)   a recombinant RNA molecule capable of expressing in the cells the
              alphavirus structural glycoprotein;

       producing said alphavirus replicon particles in the cells, and collecting said
       alphavirus replicon particles from the cells.

64.   A method for making infectious, replication-defective alphavirus replicon
       particles, comprising introducing into a population of cells the following:

       (a)   an alphavirus structural protein expression system according to claim 54,
       and
       (b)   an alphavirus replicon RNA encoding at least one heterologous RNA;

wherein the alphavirus structural protein expression system expresses all the alphavirus structural proteins, producing said alphavirus replicon particles in the cells, and collecting said alphavirus replicon particles from the cells.

65. The method according to claim 63 or 64, wherein the alphavirus structural proteins are selected from the group consisting of VEE, Sindbis, S.A.AR 86, Semliki Forest Virus and Ross River Virus.

ATTC

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<110> AlphaVax, Inc.
      Jonathan F. Smith
      Kurt I. Kamrud
      Jonathan O. Rayner
      Sergey A. Dryga
      Ian J. Caley

<120> ALPHAVIRUS REPLICON VECTOR SYSTEMS

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cgggatccat gcggtttgat gcgggtgcat acatc   35

<210> 34
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 34
gctctagatt agccgtagag agttataggg g   31

<210> 35
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 35
tggcgcgccg ctcggaattc cccctctccc   30

<210> 36
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

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<400> 36
aggcgcgcct tctatgtaag cagcttgcc                                        29

<210> 37
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
     Synthetic Construct

<400> 37
ccgggaaaac agcattccag gtattaga                                         28

<210> 38
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
     Synthetic Construct

<400> 38
tttttttttt tttttttttt tttttttttt tttttttttt tttttgaaat attaaaaaca     60
aaatccgatt cgg                                                         73

<210> 39
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
     Synthetic Construct

<400> 39
gacgggcccc ttgcccttcg tagcgacac                                        29

<210> 40
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
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<400> 40
gacgggccca gtttccaggt cagggtcgc                                        29

<210> 41
<211> 33
<212> DNA
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 41
gacgggcccc cttcattttc ttgtccaatt cct   33

<210> 42
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 42
gacgggccct gcatacttat acaatctgtc cgga   34

<210> 43
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
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<400> 43
gacgggcccc ggacagatac aatgatactt gtgct   35

<210> 44
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 44
gacgggcccg ccagatgcga aaacgctctg   30

<210> 45
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 45
gacgggcccg ccagatgcga aaacgctctg   30

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<210> 46
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 46
gacgggccct acctcaaact gcgggaagc   29

<210> 47
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
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<400> 47
gacgggccct tttgggtagg taattggtct gg   32

<210> 48
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
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<400> 48
gacgggcccc tataactctc tac   23

<210> 49
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence; NOTE =
      Synthetic Construct

<400> 49
gcaacgcggg gaggcagaca   20

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 7/02, 15/86, 5/10
US CL : 424/93.2, 435/69.1, 320, 325, 455, 456, 457

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/93.2, 435/69.1, 320, 325, 455, 456, 457

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HERWEIJER et al. Self-ampifying vectors for gene delviery. Advanced Drug Delivery Reviews. 1997, Vol. 27, pages 5-16, especially pages 7-10.	1-65
Y	US 6,329,201 B1 (POLO et al.) 11 December 2001 (11.24.2001), see column 3 trhough column 6, column 16, line 20-56 column 18, line 30-64, and example 1-7.	1, 3, 4, 5, 8, 18, 19, 20, 22, 23, 24, 26, 27, 28, 29, 32- 47, 50.
Y	LEE et al. Efficient Long-Term Coexpression of a Hammerhead Robozyme Targeted to the U5 Region of HIV-1 LTR by Linkage to the Multidrug-Resistance Gene. Antisense and Nucleic Acid Drug Development. 1997, Vol. 7, pp 511-522.	1-65

☐ Further documents are listed in the continuation of Box C.	☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 January 2003 (27.01.2003)	11 FEB 2003
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Maria B Marvich, PhD Telephone No. (703) 308-1235

PCT/US02/28610

Continuation of B. FIELDS SEARCHED Item 3:
East databases USPAT, PGPUB, EPO, JPO, Derwent, IBM-IDB
STN databases Medline, CAPLUS, Scisearch